FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV. 10.95)

ATTORNEY'S DOCKET NUMBER
674537-2001

09/581924
U.S APPLICATION NO. (If known see 37 C.F.R. 1 5)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	(EARLIEST) PRIORITY DATE CLAIMED
PCT/AU98/01076	**24 DECEMBER 1998**	**24 DECEMBER 1997**

TITLE OF INVENTION: **BIFUNCTIONAL MOLECULES**

APPLICANTS FOR DO/EO/US: **John Leslie ATWELL, Peter Leonard DEVINE, Gregory COIA, Alexander Andrew KORTT, Gillian Wendy PERRY and Peter Gregory BUNDESEN**

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)), including 11 sheets of formal drawings and a copy of the International Search Report.
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
10. ☐ The annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

    PCT/ISA/210, PCT/RO/101,

    PCT/IPEA/409,

    References for IDS, Sequence listing (3 sheets)

    1 Page Abstract

EXPRESS MAIL

Mailing Label Number: EL560704765US

Date of Deposit: June 19, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT Washington, DC 20231.

Charles Jackson
(Typed or printed name of person mailing paper or fee)

Charles Jackson
(Signature of person mailing paper or fee)

JMM2518

U.S. APPLICATION NO.(If known, see 37 C.F.R. 1.50) 09/581924 | INTERNATIONAL APPLICATION NO. PCT/AU98/01076 | ATTORNEY'S DOCKET NO. 674537-2001

17. ☒ The following fees are submitted: (CALCULATIONS /PTO USE ONLY

Basic National Fee (37 CFR 1.492(a)(1)-(5):

Search Report has been prepared by the EPO or JPO.....$840.00 ($840.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) $700.00

No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))...$770.00

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37CFR 1.445(a)(2) paid to USPTO.....$1040.00

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT = **($ 840.00**

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)). ($ /

89 Claims	/Number Filed /	Number Extra	/Rate (
Total Claims	/ 89 - 20 = /	69	/X $18.00	**($1,242.00**
Independent Claims	/ 5- 3 = /	2	/X $78.00	**($ 390.00**
Multiple dependent claim(s) (if applicable)			/+ $260.00	($

TOTAL OF ABOVE CALCULATIONS = ($2,472.00

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28). (/ **($ 0.00/**

SUBTOTAL = **($2,472 .00**

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).+ (/ ($ /

TOTAL NATIONAL FEE = ($2,472.00

Fee for recording the enclosed assignments (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **$40.00** per property + **($ 40.00/** ($ /

TOTAL FEES ENCLOSED = ($2,512.00

(Amount to be: / (refunded /$

(charged /$

a. ☒ Our checks in the amount of $2,512.00 are cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 50-0320 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0320**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

THOMAS J. KOWALSKI
FROMMER LAWRENCE & HAUG LLP
745 FIFTH AVENUE
NEW YORK, NEW YORK 10151

Dated: June 19, 2000

Thomas J. Kowalski
SIGNATURE

THOMAS J. KOWALSKI
NAME

32,147
REGISTRATION NUMBER

Form PTO-1390 (REV **10-96**)

JMM2518

09 / 581924

PATENT

674537-2001

534 Rec'd PCT/PTO 19 JUN 2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	ATWELL et al.
U.S. Serial No.	:	Filed Concurrently Herewith
Int'l Appln. No.	:	PCT/AU98/01076
Int'l Filing Date	:	24 DECEMBER 1998
Earliest Priority Date	:	24 DECEMBER 1997
For	:	BIFUNCTIONAL MOLECULES

745 Fifth Avenue
New York, NY 10151

EXPRESS MAIL

Mailing Label Number: EL560704765US

Date of Deposit: June 19, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

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Charles Jackson

(Signature of person mailing paper or fee)

09581924.061900

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Box PCT
Washington, D.C. 20231

Dear Sir:

Preliminary to the examination of this U.S. national phase application, please enter the following amendments:

IN THE SPECIFICATION:

Page 1, below "Bifunctional molecules" please insert:

--RELATED APPLICATIONS

This application is the National Phase of PCT/AU98/01076, filed December 24, 1998, designating the U.S. and published as WO 99/33965, with claims of priority from Australian application nos. PP1110 and PP5176, filed December 24, 1997 and August 11, 1998. All of the foregoing applications, as well as all documents cited in the foregoing applications ("application documents") and all documents cited or referenced in application documents are hereby incorporated herein by reference. Also, all documents cited in this application ("herein cited documents") and all documents cited or referenced in herein cited documents are hereby incorporated herein by reference.

IN THE CLAIMS:

Claim 3, line 1, please delete "or claim 2" and insert --claim 1--;

Claim 4, line 1, please delete "any one of claims 1 to 3" and insert --claim 1--;

Claim 5, line 1, please delete "any one of claims 1 to 4" and insert --claim 1--;

Claim 6, line 1, please delete "any one of claims 1 to 5" and insert --claim 1--;

Claim 8, line 1, please delete "any one of claims 1 to 5" and insert --claim 1--;

Claim 10, line 1, please delete "any one of claims 1 to 5" and insert --claim 1--;

Claim 11, line 1, please delete "any one of claims 1 to 10" and insert --claim 1--;

Claim 12, line 1, please delete "any one of claims 1 to 11" and insert --claim 1--;

Claim 15, lines 1-2, please delete "or claim 14";

Claim 16, lines 1-2, please delete "any one of claims 13 to 15" and insert --claim 13--;

Claim 18, lines 1-2, please delete "any one of claims 13 to 17" and insert --claim 13--;

Claim 22, lines 1-2, please delete "or claim 21";

Claim 23, lines 1-2, please delete "any one of claims 20 to 22" and insert --claim 20--;

Claim 24, lines 1-2, please delete "any one of claims 20 to 23" and insert --claim 20--;

Claim 26, lines 1-2, please delete "any one of claims 20 to 25" and insert --claim 20--;

Claim 28, lines 1-2, please delete "any one of claims 20 to 27" and insert --claim 20--;

Claim 29, lines 1-2, please delete "any one of claims 13 to 29" and insert --claim 13--;

Claim 33, lines 4-5, please delete "any one of claims 1 to 12" and insert --claim 1--;

Claim 35, line 1, please delete "or claim 34";

Claim 36, line 1, please delete "any one of claims 33 to 35" and insert --claim 33--;

Claim 37, line 1, please delete "any one of claims 33 to 35" and insert --claim 33--;

Claim 38, line 1, please delete "any one of claims 33 to 35" and insert --claim 33--;

Claim 43, line 1, please delete "any one of claims 39 to 42" and insert --claim 39--;

Claim 44, line 1, please delete "any one of claims 39 to 43" and insert --claim 39--;

Claim 49, line 1, please delete "any one of claims 39 to 43" and insert --claim 39--;

Claim 51, line 1, please delete "any one of claims 39 to 50" and insert --claim 39--;

Claim 52, line 1, please delete "any one of claims 39 to 51" and insert --claim 39--;

Claim 54, line 1, please delete "any one of claims 39 to 51" and insert --claim 39--;

Claim 56, line 1, please delete "any one of claims 39 to 51" and insert --claim 39--;

Claim 57, line 1, please delete "any one of claims 39 to 56" and insert --claim 39--;

Claim 58, line 1, please delete "any one of claims 39 to 56" and insert --claim 39--;

Claim 59, line 1, please delete "any one of claims 39 to 58" and insert --claim 39--;

Claim 60, line 2, please delete "any one of claims 39 to 59" and insert --claim 39--;

Claim 68, line 1, please delete "any one of claims 65 to 67" and insert --claim 65--;

Claim 73, line 1, please delete "any one of claims 65 to 67" and insert --claim 65--;

Claim 75, line 1, please delete "any one of claims 65 to 74" and insert --claim 65--;

Claim 77, line 1, please delete "any one of claims 65 to 74" and insert --claim 65--;

Claim 79, line 1, please delete "any one of claims 65 to 74" and insert --claim 65--;

Claim 80, line 1, please delete "any one of claims 65 to 79" and insert --claim 65--;

Claim 81, line 1, please delete "any one of claims 65 to 79" and insert --claim 65--;

Claim 82, line 1, please delete "any one of claims 65 to 81" and insert --claim 65--;

Claim 83, line 1, please delete "any one of claims 65 to 82" and insert --claim 65--;

Claim 84, line 4, please delete "any one of claims 65 to 83" and insert --claim 65--;

Claim 86, line 1, please delete "or claim 85";

Claim 87, line 1, please delete "any one of claims 84 to 86" and insert --claim 84--;

Claim 88, line 1, please delete "any one of claims 84 to 86" and insert --claim 84--; and

Claim 89, line 1, please delete "any one of claims 84 to 86" and insert --claim 84--.

IN THE ABSTRACT:

Please add the Abstract attached hereto as a separate sheet.

REMARKS

This application includes multiple claim dependencies. The amendment removes the multiple claim dependencies, and the filing fee for this application was computed on the basis that no dependent claim depends from more than one preceding claim. The amendment also adds headings and other formalities.

Entry of this amendment and an early examination on the merits are respectfully solicited.

Respectfully submitted,
FROMMER LAWRENCE & HAUG LLP

By: [signature]
Thomas J. Kowalski
Reg. No. 32,147
(212) 588-0800

ABSTRACT

The invention relates to a chimeric antibody conjugate comprising an antigen binding region of a non-human antibody and an immunoglobulin constant region which comprises at least one C_H domain or epitope thereof, with the proviso that the constant region is not a naturally occurring F_C fragment. A bifunctional molecule for use in labelling an antibody derived from a first species, the bifunctional molecule comprising a binding region which binds to the antibody of the first species or to one or more groups provided thereon, and a constant region derived from an antibody of a second species, the constant region comprising at least one C_H domain or an epitope thereof. The present invention relates to bifunctional molecules and complexes which are useful as positive control reagents in antibody based diagnostic tests. The present invention also relates to polynucleotides encoding these bifunctional molecules, and to diagnostic assays involving the use of these molecules.

Bifunctional Molecules

Field of the Invention

The present invention relates to bifunctional molecules and complexes which are useful as a positive control reagents in antibody based diagnostic tests. The present invention also relates to polynucleotides encoding these bifunctional molecules, and to diagnostic assays involving the use of these molecules.

Background of the Invention

Infection of humans by many micro-organisms leads to the initiation of a humoral immune response that can be used in the diagnosis of the disease. In the early acute phase of the infection, specific IgM class antibodies are the first to appear in serum 1-4 weeks after the onset of symptoms and last for up to three months. IgG class antibodies appear later and remain elevated throughout the patient's life. Detection of an IgM response is indicative of a recent or current infection, while the presence of an elevated IgG response is a marker for past exposure to the causative agent. Specific IgM or IgG responses to a particular infectious agent can be measured by antibody based diagnostic tests such as ELISA, immunochromatography, particle agglutination ELISA, biosensor or other similar assays.

These assays require the use of reactive human sera as a positive control. The positive control reagent is usually serum taken from a patient or animal which is known to have a positive reaction to the particular antigen under test. If the test is designed to distinguish between early and late infection (via the differentiation between immunoreactive IgM, for early infection and IgG, for late or previous infection), the positive control serum or reagent should contain immunoreactive antibody of the correct immunoglobulin class.

It is becoming increasingly difficult to source sufficient quantities of immune human sera or plasma, particularly as diagnostic tests for rarer diseases become available. Collection of blood for IgM controls from patients in early stages of infection when clinical symptoms are generally most severe poses significant ethical problems, particularly if the disease primarily affects juveniles. Other drawbacks include the requirement for consistent collections from remote locations, the need to standardise each batch and to

check for contamination with infectious agents such as HIV, hepatitis B and hepatitis C. There are also problems in obtaining control sera for specific endemic diseases in communities where the donation of blood or blood products is socially unacceptable.

There is therefore a need for a source of positive control reagents which does not rely on being obtained from human donors.

Hybridoma technology provides a plentiful supply of monoclonal antibodies, but as these are generally of murine origin, they do react with binding reagents used to quantify human antibodies. Intact, functional mouse/human chimeric antibodies have been described in the literature for some time (Boulianne et al., 1984, Morrison et al., 1984; Winter et al., 1991). In these constructs the antigen binding function residing in a mouse Fab or Fv fragment has been grafted on to a human Ig backbone and expressed in hybridoma cells. In some cases these reshaped molecules have been designed for human therapy, utilising the effector functions of the human Fc for targeting (Reichmann et al., 1988). Others have been designed as positive control reagent substitutes (Hamilton, 1990, 1991), where V_H and V_L regions from a mouse monoclonal antibody of desired specificity have been grafted onto either a human IgG or IgM backbone.

Synthetic positive control reagents are available from a limited number of sources. US 4,929,543 relates to chimeric antibody fragments where Fab or F(ab')2 fragments of non human origin, with specificity for the desired antigen, are chemically coupled to human Fc fragments in order to confer upon the reactive non-human Fab fragments epitopes recognised by class specific anti human immunoglobulin antisera. This reference does not teach or suggest coupling non-human Fab or F(ab')2 fragments to individual C_H domains in order to provide epitopes for recognition by class specific anti human immunoglobulin antisera. Furthermore, production of the chimeric fragments is entirely by synthetic routes based upon digestion of antibodies, purification of fragments and chemical linking to create the chimera.

Labor Diagnostika GmbH of Heiden, Germany have produced synthetic positive control reagents which are formed by chemical attachments of non human Fab fragments and human Fc fragments onto a latex bead. These attachments confer upon the bead the twin properties required of a positive control reagent - specific antigen binding and human immunoglobulin class specific epitopes.

A process for producing positive control reagents which circumvents the requirement to manipulate full length Fc fragments, or to manipulate VH and VL sequences for each new control reagent specificity, is desirable.

Summary of the Invention

The present inventors have now developed bifunctional molecules which may be used as positive control reagents in antibody based diagnostic tests.

In one aspect of the present invention, the bifunctional molecule is a chimeric antibody conjugate comprising a first region which binds a specific antigen and a second region comprising at least one constant domain sequence derived from a class specific immunoglobulin. This conjugate, which may be used directly as a positive control reagent, avoids the inconvenience of manipulating full length or naturally occurring Fc fragments. Furthermore, the conjugate may be readily produced by recombinant DNA technology.

Accordingly, in a first aspect the present invention provides a chimeric antibody conjugate comprising an antigen binding region derived from a non-human antibody and a constant region which comprises at least one C_H domain or epitope thereof, with the proviso that the constant region is not a naturally occurring F_C fragment.

When used herein, "naturally occurring Fc fragment" means a full length naturally occurring Fc fragment which may be derived by proteolytic digestion of an intact antibody molecule. For example, a naturally occurring Fc fragment of IgM will comprise domains C_H2, C_H3 and C_H4, whereas a naturally occurring IgG Fc fragment will comprise C_H2 and C_H3 domains.

By "chimeric" we mean that the constant region is derived from a different species than the antigen binding region.

In a preferred embodiment the non-human antigen binding region comprises or consists of a non-human Fab fragment or part thereof. The non-human antigen binding region may comprise or consist of an scFv fragment.

In a further preferred embodiment the non-human antigen binding region is derived from a mouse.

In a preferred embodiment the constant region is derived from a human antibody. It will be appreciated, however, that the constant region may be a non-human (such as bovine, canine, ovine, equine, feline or

caprine) constant region in cases where the chimeric construct is to be used as a positive antibody control in assays involving sera derived from non-human species.

The constant region may consist of a non-naturally occurring combination of C_H domains or epitopes thereof. The constant region may consist of two C_H domains of the same type, for example, two C_H3 domains. Alternatively, the constant region may consist of two different domains. The two different domains, or epitopes thereof, may be derived from antibodies of different classes. In a preferred embodiment, the constant region consists of a single C_H domain.

In a particularly preferred embodiment of the present invention the chimeric antibody conjugate is suitable as a positive IgM control and the constant region comprises one or more $C_H3\mu$ domains.

In a further preferred embodiment the non-human antigen binding region binds to an epitope derived from an infectious agent selected from but not limited to dengue virus, rubella virus, herpes virus, parvovirus, human glycophorin, *Rickettsia sibirica, Burkholeria pseudomallei, Salmonella typhi* or *paratyphi, Leptospira interrogans, Plasmodium falciparum/vivax,* Japanese encephalitis virus, Yellow fever virus, *Bordetella pertussis/parapertussis, Candida albicans/kruzei,* Varicella zoster virus, HIV, Hepatitis viruses, Human papilloma virus, Epstein-Barr virus, Ross River virus, *Brucella abortis,* Human herpesvirus-6, Parvovirus B19, *Coxiella burnettii,* Herpes simplex viruses 1&2, *Rickettsia rickettsii, Conori australis, Rickettsia tsutsugamushi.*

In a second aspect the present invention provides a recombinant polynucleotide molecule comprising a sequence encoding a non-human V_H region, a sequence encoding a non-human V_L region, a sequence encoding a flexible linker positioned between the V_H region sequence and the V_L region sequence, and a heterologous sequence encoding a C_H domain or epitope thereof.

By "heterologous sequence encoding a C_H domain" we mean sequence encoding a C_H domain which is derived from a different species than the sequences encoding the V_H and V_L regions.

In a preferred embodiment of the second aspect the heterologous sequence encodes a human C_H domain.

By 'flexible linker' we mean a region of amino acids of sufficient length and flexibility to allow the V_H and V_L polypeptide regions to dock

correctly with respect to each other to form an scFv fragment. The flexible linker may be a polypeptide of between 12 and 30 amino acids in length. Preferably the linker is a polypeptide of about 15 amino acids in length. The linker may have the sequence GGGGSGGGGSGGGGS.

In a preferred embodiment. the C_H domain sequence is linked to the 3' end of the V_L or V_H sequence. In this preferred construct the chimeric antibody conjugate is synthesized as a single polypeptide chain which folds to produce two separate functional domains.

In a further preferred embodiment of the second aspect of the invention, the polynucleotide molecule includes a control sequence which directs the synthesis of both the V_L and V_H polypeptide regions. The control sequence is preferably an inducible promoter such as the lac promoter.

In a further preferred embodiment the polynucleotide molecule includes a sequence encoding a leader peptide which directs the synthesised polypeptide chains to the host cell periplasm. The leader sequence may be the pel B sequence.

In a third aspect the present invention provides a recombinant polynucleotide molecule comprising a sequence encoding a non-human V_L region, a sequence encoding a non-human C_L region, a sequence encoding a non-human V_H region, a heterologous sequence encoding a C_H domain or epitope thereof and optionally a sequence encoding a non-human C_H1 region.

In a preferred embodiment of the third aspect the heterologous sequence encodes a human C_H domain.

In a further preferred embodiment of the third aspect of the present invention, the V_L and C_L sequences are linked together so that the V_L and C_L regions are expressed as a single polypeptide. In a more preferred embodiment, the V_H and C_H1 sequences are also linked together so that the V_H and C_H1 regions are expressed as a single polypeptide.

In a further preferred embodiment of the third aspect the polynucleotide molecule includes a control sequence which directs the synthesis of both the V_L-C_L and V_H-C_H1 polypeptide chains. The control sequence is preferably an inducible promoter such as the lac promoter.

In a further preferred embodiment of the third aspect the polynucleotide molecule includes a sequence encoding a leader peptide which directs the synthesised polypeptide chains to the host cell periplasm. The leader sequence may be the pel B sequence. Preferably, the V_L-C_L and

V_H-C_H1 polypeptide chains associate in the host cell periplasm and are stabilised by one or more disulphide bonds between the chains.

In a further preferred embodiment of the third aspect the heterologous C_H domain sequence is linked to the V_L-C_L sequences or the V_H-C_H1 sequences so that the expressed heterologous C_H domain is attached to the V_L-C_L polypeptide or the V_H-C_H1 polypeptide.

In a further preferred embodiment of the third aspect the non-human C_H1 sequence is absent from the recombinant polynucleotide construct. The heterologous C_H domain sequence may be linked directly to the non-human V_H sequence to give rise to a chimeric non human V_H-human C_H polypeptide chain. This chimeric polypeptide chain may associate with the non-human V_L-C_L polypeptide chain to form a chimeric Fab fragment. It will be appreciated that such a chimeric Fab fragment will possess a specific antigen binding region, and a human constant region which provides a recognition site for class specific anti immunoglobulin antibodies.

The polynucleotide molecules of the second or third aspects of the present invention may be incorporated into plasmids or expression vectors which may then be introduced into suitable bacterial, yeast, insect or mammalian host cells.

Accordingly, in a fourth aspect the present invention provides a vector comprising a polynucleotide according to the second or third aspects of the present invention.

In a fifth aspect the present invention provides a bacterial. yeast, insect or mammalian host cell transformed with a vector according to the fourth aspect of the present invention.

In a sixth aspect the present invention provides a method of producing a chimeric antibody conjugate which comprises culturing a host cell according to the fifth aspect of the present invention under conditions enabling the expression of the conjugate and optionally recovering the conjugate.

In a seventh aspect the present invention provides a chimeric antibody conjugate produced by a method according to the sixth aspect of the present invention.

In yet another aspect of the present invention. the bifunctional molecule is able to bind to antibodies or antibody-like molecules and thereby label them with epitopes from immunoglobulin constant regions derived

from different species. The complex thus formed has the properties of a specific positive antibody control: a ligand binding site with specificity for the antigen, hapten or drug in question and epitopes or domains which are recognised by immunoglobulin binding reagents. The bifunctional molecules of this aspect of the invention may be produced by recombinant DNA technology. Alternatively, recombinant fragments may be linked by conventional chemical coupling technologies.

Accordingly, in an eighth aspect the present invention provides a bifunctional molecule for use in labelling an antibody of a first species, the bifunctional molecule comprising a binding region which binds to the antibody of the first species or to one or more groups provided thereon, and a constant region derived from an antibody of a second species, the constant region comprising at least one C_H domain or an epitope thereof.

The order of the binding and constant regions on the bifunctional polypeptide is not critical. The order may be either (N terminus)—binding region—constant region—(C terminus) or vice versa. ie (N terminus)—constant region — binding region —(C terminus).

In a ninth aspect the present invention provides a complex formed between (i) an antibody or biologically active fragment thereof derived from a first species and (ii) a bifunctional molecule, the bifunctional molecule comprising a binding region which binds to the antibody of the first species or to one or more groups provided thereon, and a constant region derived from an antibody of a second species, the constant region comprising at least one C_H domain or an epitope thereof.

By "biologically active fragment" we mean a fragment which mimics the binding of the antibody derived from the first species to at least one antigenic determinant.

In a preferred embodiment of the eighth and ninth aspects, the binding and constant regions of the bifunctional molecule are separated by a linker molecule. The linker molecule may be a short peptide. Preferably, the linker molecule is a peptide of between 1 and 20 amino acids in length, more preferably between 1 and 10 amino acids in length, and more preferably between 2 and 5 amino acids in length.

In a further preferred embodiment of the eighth and ninth aspects, the binding region is not derived from an antibody. By this we mean that the

binding region is preferably not (i) a Fab fragment, (ii) a portion of a Fab fragment, (iii) an ScFv fragment or (iv) a portion of an ScFv fragment.

In one embodiment of the eighth and ninth aspects, the binding region binds directly to the antibody derived from the first species.

In a further preferred embodiment of the eighth and ninth aspects, the binding region is derived from a protein selected from the group consisting of, *Streptococcal* protein G (described in Björck and Kronvall (1984), and Boyle and Reis (1987), the entire contents of which are incorporated herein by reference) *Staphylococus aureus* protein A (described in Uhlen et al. (1984), and Boyle and Reis (1987), the entire contents of which are incorporated herein by reference) and *Peptostreptococcus magnus* protein L (which is described in Åkerström and Björck (1989), the entire contents of which is incorporated herein by reference). In a further preferred embodiment, the binding region comprises one of the immunoglobulin binding regions of *Staphylococcus aureus* protein A. The immunoglobulin binding region of *Staphylococcus aureus* protein A may be fragment B.

In a further preferred embodiment of the eighth and ninth aspects, the binding region comprises a mouse Fc γ receptor or fragment thereof. The mouse Fc γ receptor may be selected from the group consisting of FcγRI, which specifically binds monomeric mouse IgG2a: FcRII, which binds aggregated IgG1, IgG2a and IgG2b; and FcγRIII, which binds the minor subclass IgG3 (see Heusser et al., 1977; Segal et al., 1978; Unkeless et al., 1988; Hogarth et al., 1987; Kulczycki et al., 1990, the entire contents of which are incorporated herein by reference).

In another preferred embodiment of the eighth and ninth aspects, the binding region comprises a histidine rich glycoprotein (as described in Borza et al., 1996 and Gorgani et al., 1997, the entire contents of which are incorporated herein by reference).

In another embodiment of the eighth and ninth aspects, the binding region binds to one or more groups provided on the antibody of the first species. Preferably, the group(s) is a biotin molecule and the binding region comprises streptavidin (described in Argaraña et al. (1986), US 5672691 and US 5489528, the entire contents of which are incorporated herein by reference) or a fragment thereof.

In a further preferred embodiment of the eighth and ninth aspects of the present invention, the first species is a rat or a mouse.

In a further preferred embodiment of the eighth and ninth aspects, the antibody of the first species is a monoclonal antibody. In a further preferred embodiment, the antibody of the first species is an IgG antibody.

In a further preferred embodiment of the eighth and ninth aspects, the antibody constant region is not a naturally occurring Fc fragment.

In a further preferred embodiment of the eighth and ninth aspects, the antibody constant region comprises or consists of a non-naturally occurring combination of immunoglobulin C_H domains or epitopes thereof. The constant region may include or consist of two C_H domains of the same type, for example, two $C_H3\mu$ domains. Alternatively, the constant region may include or consist of two different domains. The two different domains, or epitopes thereof, may be derived from antibodies of different classes. In a preferred embodiment, the constant region consists of a single C_H domain.

In a further preferred embodiment of the eighth and ninth aspects, the second species is a human. It will be appreciated, however, that the second species may be non-human (for example, bovine, canine, ovine, equine, feline or caprine) in cases where the bifunctional molecule or complex is to be used as a positive control reagent in assays involving sera derived from non-human species.

In a particularly preferred embodiment of the ninth aspect of the present invention, the bifunctional molecule is suitable for combination with mouse IgG as a positive IgM control and the constant region comprises one or more $C_H3\mu$ domains.

In a particularly preferred embodiment of the ninth aspect of the present invention, the bifunctional molecule is bound to a location on the antibody (or fragment thereof) of the first species which does not significantly hinder the binding between the antibody (or fragment thereof) and its specific antigen.

In a further preferred embodiment of the complex according to the ninth aspect, the affinity between the binding region and the antibody or biologically active fragment thereof derived from the first species is sufficient to form a stable complex in solution. Preferably, the binding region has a K_D for the antibody of less than 10^{-6} M. More preferably, the K_D is less than 10^{-8}M and more preferably less than 10^{-9}M.

In a further preferred embodiment of the eighth and ninth aspects, the antibody constant region is modified in order to facilitate the production

of the molecule, or to reduce aggregation of individual bifunctional molecules, without substantially altering the characteristic epitopes of the domain. For example, a cysteine residue usually associated with the formation of an inter-chain disulphide bond may be mutated to serine. In another example, a bifunctional molecule which contains a fragment of *Staphylococcal* protein A linked to a human Cγ3 domain may aggregate because of the high affinity of the protein A fragment for human IgG constant domains. This aggregation may be circumvented by a substitution His to Arg at position 435. Evidence suggests that the lack of binding of protein A to human IgG subclass 3 is related to the substitution of Arg for His at position 435 (see Deisenhofer, 1981, the entire contents of which are incorporated herein by reference).

It will be appreciated by persons skilled in the art that within the context of the present invention, the preferred C_H1 domains or epitopes will be dependent on the intended use of the bifunctional molecule. For example, if the bifunctional molecule or complex is to be used as a replacement for positive IgM control sera, the preferred C_H1 domains or epitopes will be $C_H\mu$ domains or epitopes. Alternatively, if the bifunctional molecule or complex is to be used as a replacement for positive IgG control sera, the preferred C_H domains or epitopes will be a $C_H\gamma$ domains or epitopes. If the bifunctional molecule or complex is to be used as a replacement for positive IgA control sera, the preferred C_H domains or epitopes will be a $C_H\alpha$ domains or epitopes.

In a tenth aspect, the present invention provides an isolated polynucleotide encoding a bifunctional molecule according to the eighth aspect of the present invention.

The polynucleotide molecule of the tenth aspect of the present invention may be incorporated into plasmids or expression vectors which may then be introduced into suitable bacterial, yeast, insect or mammalian host cells.

Accordingly, in en eleventh aspect the present invention provides a vector comprising a polynucleotide according to the tenth aspect of the present invention.

In a twelfth aspect the present invention provides a bacterial, yeast, insect or mammalian host cell transformed with a vector according to the eleventh aspect of the present invention.

In a thirteenth aspect the present invention provides a method of producing a bifunctional molecule which comprises culturing a host cell according to the twelfth aspect of the present invention under conditions enabling the expression of the bifunctional molecule and optionally recovering the bifunctional molecule.

In a fourteenth aspect the present invention provides a bifunctional molecule produced by a method according to the thirteenth aspect of the present invention.

In a fifteenth aspect the present invention provides a method of producing a complex according to the ninth aspect which comprises admixing an antibody or biologically active fragment thereof derived from a first species with a bifunctional molecule according to the eighth aspect of the present invention.

Methods for detecting antibodies in biological samples are well known. In general, these methods involve incubation of the sample with (i) a antigenic determinant characteristic of a particular disease, and (ii) an anti human Ig antibody. The antibody measurement is generally compared to a control measurement obtained by incubating the antigenic determinant characteristic of the disease and the anti human Ig antibody with a positive control serum obtained from an individual with the disease. The present inventors have found that the chimeric antibody conjugates of the present invention react in diagnostic tests in a manner similar to class specific positive control serum.

Accordingly, in a sixteenth aspect the present invention provides a method for detecting an antibody in a biological sample which involves comparing the level of detection obtained with the biological sample to the level of detection obtained with a positive control, wherein the positive control comprises a chimeric antibody conjugate according to the first aspect, or a complex according to the ninth aspect.

In a preferred embodiment of the sixteenth aspect of the present invention, the biological sample is a human biological sample.

In a further preferred embodiment of the sixteenth aspect of the present invention the antibodies to be detected are antibodies characteristic of a disease selected from but not limited to dengue fever, Japanese encephalitis, rubella, spotted fever, herpes infection, parvovirus infections, melioidosis, typhoid, leptospirosis, malaria, yellow fever, whooping cough,

systemic candidiasis/thrush. chicken pox, shingles. AIDS, hepatitis, liver cancer, cervical cancer. infectious mononucleosis. nasopharyngeal carcinoma, Ross River fever. brucella. exanthum subitum (Sixth disease/Roseola infantum), erythema ingfectiosum (Fifth disease). Q Fever. cold sores, genital herpes, spotted fever. scrub typhus.

The antibody to be detected in the biological sample may be an antibody of any class. In a preferred embodiment, however. the antibody is an IgM antibody.

The terms "comprise". "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated component or feature or group of components or features with or without the inclusion of a further component or feature or group of components or features.

The invention will now be described in detail by reference to the following non-limiting Figures and Examples.

Brief Description of the Figures

Figure 1 shows the structure of intact IgG antibody (a) along with the two sub fragments capable of being produced in prokaryotic or lower eukaryotic cells, Fab (b) and scFv (c). The antigen binding region. the location of the CDR loops are indicated.

Figure 2 illustrates one embodiment of the invention. The single polypeptide chain protein folds into two domains. The scFv region derived from mouse DNA sequences folds to form the specific antigen binding site. The C-domain derived from human DNA sequences of immunoglobulin constant regions folds to provide binding epitopes for heterologous. class specific anti human immunoglobulin sera.

Figure 3 shows in cartoon form the two regions of the bifunctional binding molecule and illustrates one embodiment of the invention. Also shown is the complex formed between the bifunctional molecule and intact mouse IgG in which the mouse IgG is decorated with antibody C domains, preferably from human immunoglobulin heavy chains.

Figure 4 shows a further embodiment of the invention in which a complex is formed between the bifunctional molecule and one or more groups introduced onto the antibody of species A. In the embodiment

illustrated, the group is biotin, the first region which binds to the group is streptavidin or a fragment thereof and the antibody is mouse IgG.

Figure 5 illustrates in cartoon form the structure of the bacterial expression plasmid containing the sequence of one embodiment of the invention. The expression cassette contains a chemically inducible promoter, lac, followed by sequence encoding the components of the chimeric protein. The pel B sequence directs the synthesised protein to the cell periplasm, the V_H and V_L regions joined by linker sequence are the mouse scFv antibody, and C_H is the human immunoglobulin constant domain sequence inserted between the Not I and Sac II sites. FLAG is an octapeptide recognition sequence recognised by the antibody anti FLAG® M2 (Eastman Kodak Co., New Haven, CT) used for monitoring expression; stop designates a stop codon and TT designates a transcription terminator. The remainder of the vector is derived from vector pUC19 (Yanisch-Perron et al., 1985). The entire vector minus the specific inserts is denoted pGC (Coia et al., 1996)

Figure 6 illustrates the reactivity in ELISA of the 4 different human IgM C_H domain constructs linked to the scFv 1C3 (anti-glycophorin). With glycophorin bound to the ELISA well, samples containing the gene product were introduced and incubated for 1 hour at RT. After extensive washing, wells were probed with polyclonal anti human IgM antiserum raised in sheep and labelled with horseradish peroxidase. After 1 hour incubation at RT and extensive washing, ABTS was added for colour development which was read at 405 nm after 20 mins.

Figure 7 illustrates the reactivity in a dengue IgM capture ELISA of unfractionated periplasm taken from an expression culture of the 13C11(anti dengue)scFv-$C_H3\mu$ domain construct. Positive, negative and calibrator controls were supplied with the test kit (see text) and used as directed: (100 µl per assay) at a dilution of 1/100 in serum diluent. Other reagents were as supplied with the kit. Periplasm was diluted 1/5, 1/10, 1/50 and 1/100. 100 µl of each dilution including undiluted periplasm were added to test wells on the ELISA plate, covered and incubated for 1 hour at 37°C. After 4 washes with diluted wash buffer, 100 µl of a combined mixture of stablized dengue virus antigens with peroxidase labelled anti-dengue monoclonal antibody were added and the plate further incubated for 1 hour at 37°C. After 6 washes with diluted wash buffer, 100 µl TMB solution was added and colour

development proceeded for 10 mins. 100 µl Stop solution was then added and the colour read at 450nm.

Negative, Calibrator and Positive refer to serum controls supplied with the test. Peri, peri 1/5-peri 1/100 refer to test samples containing the 13C11(anti dengue)scFv- $C_H3\mu$ domain construct. Peri neg control is derived from a culture of 1C3(antiglycophorin)- $C_H3\mu$ domain chimera (Example 1).

Figure 8 shows the DNA sequence of the expression cassette (from pGC vector) containing fragment B from *Staphylococcus aureus* Protein A joined via a short linker to the human IgM heavy chain $C_H3\mu$ domain. together with a terminal FLAG® tag sequence, pel B leader and trp A terminator.

Figure 9 shows in cartoon form the constituents of the ELISA used to demonstrate the binding of various mouse IgG subclasses to the bifunctional linking reagent described in Example 1 which has bound to immobilised polyclonal anti human IgM antibody, raised in sheep.

Figure 10 shows the verified sequence of an expression cassette in pGC comprising the pel B leader sequence, core streptavidin, human IgM C_H3 domain and FLAG® tag.

Figure 11 shows results from size exclusion chromatography on Superdex200 of a sample containing refolded Streptavidin-$C_H3\mu$ in phosphate buffered saline. Flow rate was 0.5ml/min.

Detailed Disclosure of The Invention

In one aspect the present invention relates to a recombinant chimeric antibody molecule. One region of this chimeric molecule comprises an antigen binding region derived from an antibody.

Fragments of antibody molecules containing predominantly antigen binding regions have been synthesized using prokaryote or lower eukaryote expression systems (eg bacterial or yeast cells) (see, for example, PCT/AU93/00491, the entire disclosure of which is incorporated herein by reference). The antigen binding site is composed of amino acid residues formed in up to six surface loops at the extremity of the molecule. The loops on the outer domain are termed complementarity determining regions (CDRs) and provide the specificity of binding of the antibody to the antigenic target, by variation in the amino acid composition of these surface loops. The antigen binding regions of both intact IgG and sub-fragments are illustrated in Figure 1.

In order to stabilise the paired associations of the V_H and V_L regions produced by such cultures, these regions may be expressed as one continuous polypeptide chain where there is a region of amino acids of sufficient length and flexibility interspersed between the C-terminus of one domain and the N-terminus of the other to allow the two domains to dock correctly with each other to correctly position the CDR loops. Methods of manufacture of covalently linked single chain Fv fragments are disclosed in US-4.946,778, US-5,132,405 and WO 94/07921 the entire contents of which are incorporated herein by reference.

Alternatively, the antigen binding domains can be produced as Fab fragments where two polypeptide chains V_H - C_H1 and V_L - C_L are synthesised separately from mouse gene sequences and the subsequent formation of heavy and light chain fragment pairs are stabilised by a disulphide bond between the two chains. (See, for example, Better et al., 1988, Skerra, 1993, Dolezal et al., 1995, the entire contents of which are incorporated herein by reference). A preferred source of paired V_H and V_L genes for the formation of antigen binding domains is cDNA prepared from mRNA isolated from mouse monoclonal antibody cell lines.

In one preferred embodiment the chimeric antibody conjugate is a polypeptide chain which, when expressed in *E. coli*, yeast or mammalian cells from a single gene construction, folds to produce two separate functional domains, as shown in Figure 2. The first domain binds a specific antigen, and the second domain contains a specific immunoglobulin constant domain sequence (epitope) which may be recognised and bound by antibodies, prepared in rabbits, sheep or other such animal, by immunisation with class specific human immunoglobulins.

The first functional domain may consist of an antigen binding domain, formed by paired antibody V_H and V_L regions either a) linked in active conformation via a flexible peptide linker as in a scFv molecule or b) linked to mouse C_H and C_L domains as in a Fab antibody fragment. The flexible linker used to link the V_H and V_L regions as in a scFv molecule may be a polypeptide of between 12 and 30 amino acids in length (Huston *et al.*, 1991). The V_H and V_L gene sequences which code for the antibody V_H and V_L regions may be amplified via PCR from cDNA of non-human origin (usually prepared from a mouse monoclonal antibody cell line producing antibody with binding specificity for the antigen being assayed in the diagnostic test in

question). Any antigen binding specificity may be incorporated in this domain in either the Fab or scFv conformation. Preferably, a mouse monoclonal antibody cell line exists with that specificity or a V_H /V_L pair has been selected from an antibody phage library with binding specificity for that particular antigen.

The second functional domain may consist of either a single heavy chain constant domain or several in tandem which display binding sites (epitopes) for class specific polyclonal anti immunoglobulin antisera, also known as capture antibodies. The C_H region gene sequences may be amplified via PCR from cDNA prepared from mRNA isolated from peripheral blood lymphocytes. The C_H regions can be from any of the immunoglobulin heavy chain genes, (those for IgM, IgG, IgA, IgD, IgE) and the gene product from the particular C_H region is bound by the class specific anti immunoglobulin antiserum.

It will be appreciated that chimeric antibody conjugates of the present invention are capable of reacting in diagnostic tests in a manner similar to a class specific positive control serum. The chimeric conjugate will bind to a specific antigen, and will in turn be bound by the class specific capture antibodies which determine its immunoglobulin. An advantage of the conjugates of the present invention is that they may be produced in large quantities, free of contaminants, by recombinant DNA technology.

In yet another aspect the present invention relates to a bifunctional molecule which is able to bind to antibodies or antibody-like molecules and thereby label them with epitopes from immunoglobulin constant regions derived from different species. Preferably, the bifunctional molecule is a single polypeptide chain which when expressed in *E. coli*, yeast or mammalian cells folds to produce two separate functional domains, as shown in Figure 3. The first domain preferably binds to a specific region of an antibody, for example, mouse IgG, and the second domain contains a specific immunoglobulin constant domain sequence (epitope) which may be recognised and bound by antibodies, prepared in rabbits, sheep or other such animal, by immunisation with class specific human immunoglobulins.

The affinity of binding between the binding region and antibody is preferably sufficient to form a stable complex in solution between mouse IgG and the bifunctional molecule. The region on the antibody to which the bifunctional molecule binds is preferably in a location which will not

sterically hinder the binding between the mouse IgG antibody binding site and its specific antigen.

In one embodiment. the binding region binds to a group provided on the antibody. This particular embodiment is illustrated in Figure 4, in which the binding region comprises streptavidin or a fragment thereof and the antibody is mouse IgG.

The invention will be described in detail by reference to the following non-limiting examples.

EXAMPLE 1

Production of a C-domain (IgM) extended scFv

The gene sequences of the four constant domains (C domains) of human IgM heavy chain were separately amplified from cDNA prepared from mRNA isolated from human peripheral blood lymphocytes using polymerase chain reaction techniques. The design of the oligonucleotide primers used in the amplifications was based upon the 5' and 3' base sequence of each of the four IgM heavy chain exons, obtained through GENBANK accession X14940 (Dorai and Gillies, 1989).

In the primers, specific restriction enzyme recognition sequences were added (*Not*I at the 5' end and *Sac*II at the 3' end) to each exon sequence to facilitate the introduction of the C domain sequence at a specific site in a previously constructed plasmid expression vector. The expression cassette of this *E. coli* plasmid vector (pGC; Coia et al., 1996) contained V_H and V_L sequences from the mouse monoclonal antibody cell line 1C3, (Rylatt *et al.*, 1990, WO91/04492) with binding specificity for human glycophorin. These were arrayed in the scFv format, with the 3' end of the V_H sequence linked to the V_L sequence via a 45 bp sequence which coded for the protein sequence GGGGSGGGGSGGGGS. In the synthesized protein, this flexible linker region allows the correct docking of V_H with V_L. The site for the introduction of the heavy chain exon was at the 3' end of the mouse V_L sequence without any intervening sequence, save that for the restriction sites, as shown in Figure 5. Fragments were ligated together using the normal protocols and the ligation mix used to transform *E. coli* strain XL1-Blue by electroporation.

Recombinant protein was produced from positively transformed colonies of the four different constructions (each with a different IgM C-domain sequence) by induction of the plasmid lac promoter with 0.5 mM

IPTG in log phase cultures grown at 30°C. After a further 4 hours incubation at 25°C. the cell pellet was harvested by centrifugation, and the contents of the cell periplasm isolated using the protocol of Minsky et al. (1986).

The periplasmic fraction was assayed by ELISA for the presence of protein molecules with the following properties - 1) the ability to bind to immobilised glycophorin on the ELISA plate and 2) a target for the binding of horseradish peroxidase-labelled polyclonal anti human IgM serum (prepared in sheep. Silenus Laboratories. Melbourne Australia). The results in Figure 6 show that the construction with IgM constant domain 3 ($C_H3\mu$) gave the strongest reaction with the labelled polyclonal antiserum to human IgM. This was followed by domain $C_H2\mu$, with domains $C_H4\mu$ and $C_H1\mu$ showing the weakest reactions. It was a surprising observation that the majority of the reactivity of the polyclonal antiserum was directed to one domain of the IgM heavy chain. Thus, for this particular polyclonal antiserum directed against human IgM. the construct scFv-$C_H3\mu$ is a molecule with the preferred properties of a substitute for a positive human serum control.

A scFv with binding specificity for human glycophorin was solely used to demonstrate the present invention. As will be appreciated by persons skilled in this field, the antigen binding portion of the conjugate may be replaced with binding specificities to other antigenic entities which are the focus of a wide range of diagnostic test applications.

EXAMPLE 2

Construction of extended scFv (13C11 antidengue) linked to a human IgM C domain

The reagent was produced from a DNA construct in which the coding region for a mouse scFv directed against dengue virus was genetically linked to that of the third constant domain of human IgM heavy chain ($C_H3\mu$). cDNA was prepared from mRNA isolated from the mouse monoclonal antibody cell line 13C11. which specificity for Dengue virus surface antigens (Queensland University of Technology and PanBio Pty Ltd.). Immunoglobulin V_H and V_L domain DNA sequences were amplified from the cDNA using polymerase chain reaction and oligonucleotide primer sets according to Zhou et al. (1994). These were linked in the scFv format V_H - linker- V_L, where the linker was a 45 bp nucleic acid sequence coding for the protein sequence GGGGSGGGGSGGGGSGGGGS. The resultant fragment was

digested with restriction endonucleases *Nco* I and *Not* I and purified by agarose gel electrophoresis. The expression vector as described in Example 1 which contained the 1C3 (antiglycophorin) scFv- $C_H3\mu$ domain sequence was also digested with *Nco* I and *Not* I to excise the coding sequence for the 1C3 scFv. The remainder of the vector (plus C domain coding sequence) was purified and ligated with the digested 13C11 (anti dengue) scFv coding region using standard protocols. This ligated DNA was then used to transform *E. coli* strain XL1-Blue by electroporation. Positive colonies were verified both by DNA sequencing and small scale protein expression.

E. coli strain TOPP6 (Stratagene, La Jolla, CA) was transformed with purified plasmid DNA from a verified clone and used for protein expression. 500 ml of 2xYT medium (1.0 % Yeast extract, 1.6% Bacto Tryptone, 1.0 % NaCl) supplemented with ampicillin at 200 μg/ml was inoculated with an overnight culture of the transformed TOPP6 cells and incubated at 37°C with agitation until the A_{600} had reached 1.0. IPTG (isopropylthiogalactoside) was added to a concentration of 0.5 mM to induce the expression of the chimeric gene construct. The culture was shaken for a further 4 hours at a 25°C. Cells were harvested by centrifugation and the periplasmic contents isolated using the protocol of Minsky et al. (1986).

Dilutions of the unfractionated periplasm were analysed in the Dengue IgM Capture ELISA kit (PanBio Ltd, Windsor, QLD, Australia: Sang et al., 1998) using positive, negative and calibrator serum controls as supplied in the kit. In the assay, human IgM antibodies are captured by surface bound polyclonal anti human IgM antiserum (Silenus), and incubated with soluble dengue antigens plus a peroxidase labelled, dengue antigen-specific monoclonal antibody, to reveal the presence of dengue specific antibodies.

The results are shown in Figure 7. The periplasm fractions reacted positively, with the neat, 1/5 and 1/10 dilutions giving higher absorbance readings at 450 nm than the normal control. At 1/50, the periplasm still gave an absorbance reading greater than the calibrator control, which marks the cut-off between positive and negative reactions. It was calculated that an absorbance reading equivalent to the positive control would have been obtained from a periplasm dilution of 1/30. Periplasm from a culture of an unrelated chimera (1C3-$C_H3\mu$, anti-glycophorin, see Example 1) showed no positive reaction in this test.

EXAMPLE 3

Construction of Extended scFv (13C11 anti dengue) linked to a human IgG C-domain

The gene sequences of human IgG constant domains 2 and 3 were separately amplified from cDNA from mRNA isolated from human peripheral blood lymphocytes using polymerase chain reaction techniques. The design of the oligonucleotide primers used in the amplifications were based upon the 5´ and 3´ sequences for each of the heavy chain exons. obtained through Genbank accession no E06998.

Sequences coding for *Not*I and *Sac*II restriction sites were added to the 5´ and 3´ end respectively of the $C_H2\gamma$ and $C_H3\gamma$ sequences to enable the insertion into pGC 13C11-$C_H3\mu$ from which the $C_H3\mu$ sequence had been removed as a *Not*I—*Sac*II fragment.

Expression in *E. coli* and purification of product was performed as described in Example 2. The presence of product in the periplasmic fraction was confirmed by analysis of samples by polyacrylamide gel electrophoresis and Western blotting, probing the FLAG® tag using mouse anti FLAG® M2 antibody (Hopp et al., 1988) The products were denoted 13C11$C_H2\gamma$ and 13C11$C_H3\gamma$ respectively.

PanBio Indirect Dengue ELISA using 13C11CH2γ and 13C11CH3γ

Periplasmic samples containing 13C11$C_H2\gamma$ and 13C11CH3γ were diluted 1/10 in PBS /0.05% Tween 20 and 100 μl loaded in duplicate on an ELISA plate coated with dengue antigens (PanBio Dengue Indirect ELISA Kit Cat DET500) and incubated for 1 hour at 37°C. Controls included PBS/Tween 20 as negative control and positive IgG control serum which was probed with both anti human IgM and IgG antibodies. After 6 washes with PBS/0.05% Tween20, samples and controls were then probed with either sheep anti human IgM or IgG labelled with horseradish peroxidase (Silenus/AMRAD, Melbourne) at 1:1000 dilution in PBS/0.05% Tween20. The plate was incubated at 37°C for 1 hour then washed 6 times as previously. 100μl TMB reagent. supplied with the kit, was added to each well, the plate incubated at room temperature for 10 min. then the reaction was stopped by the addition of 100μl 1M phosphoric acid. Colour intensity was read at 450 nm. The results are shown in Table 1.

Table 1

	Absorbance 450nm	
	HRP-anti Human IgG	HRP-anti Human IgM
13C11 CH3γ Periplasm 1/10	1.684, 1.670	0.111. 0.109
13C11 CH2γ Periplasm 1/10	0.500. 0.586	0.133. 0.113
Human IgG (Dengue Positive) 1/100	0.971. 0.940	0.202. 0.361
PBS/0.05% Tween 20	0.308. 0.261	0.112. 0.275

Both 13C11C_H3 γ and 13C11C_H2γ extended scFvs show a positive reaction above background in this ELISA. the results with the 13C11 C_H3γ periplasm being comparable or better than the Human IgG dengue positive control. The reaction with the construct containing the human IgG C_H3γ domain gave a stronger response than with the CH2γ domain. indicating that extended scFvs with a C_H3γ domain would be the preferred construct for use as a replacement IgG positive control.

EXAMPLE 4

Production of a bifunctional molecule containing the B fragment of Staphylococcal Protein A linked to a human IgM C-domain (FB-C_H3μ)

The gene sequence for fragment B of Protein A (FB) from *Staphylococcus aureus* was amplified from chromosomal DNA prepared from strain ATCC 25923 using polymerase chain reaction techniques. The design of the oligonucleotide primers used in the amplification was based upon the 5' and 3' base sequences as reported by Uhlen et al. (1984), also GENBANK accession J01786. In the primers specific restriction enzyme recognition sites were added *Nco*I at the 5' end and *Not*I at the 3' end to facilitate the introduction at a specific site in a previously constructed plasmid expression vector (pGC; Coia et al., 1996).

In this vector the sequence encoding a human IgM C domain (C_H3μ) had previously been inserted as a *Not*I - *Sac*II fragment. A short sequence encoding the three amino acids Ser, Asp, Pro was included downstream of the FB fragment and before the *Not I* site to introduce some flexibility between the FB domain and the human C_H3μ domain. The Human C_H3μ domain had previously been amplified from cDNA prepared from mRNA

isolated from human peripheral blood lymphocytes using polymerase chain reaction techniques. using oligonucleotides based upon the 5' and 3' sequences of the domain obtained through GENBANK accession X14940 (Dorai and Gillies, 1989). We have demonstrated herein that human IgM C domain 3 ($C_H3\mu$) contains the major reactive epitopes which are bound by several polyclonal and monoclonal anti human IgM antisera capture reagents. Fragments were ligated together using standard ligation protocols and the ligation mix then used to transform *E. coli* strain XL1 Blue by electroporation. The complete DNA sequence of the expression cassette comprising the pel B leader sequence, fragment B of *S. aureus* Protein A, human $C_H3\mu$ domain, and FLAG® (a tag recognition sequence, Hopp et al., 1988) was verified by using automatic DNA sequencing methods and is shown in SEQ ID NO: 2 and Figure 8.

Recombinant protein was produced from positively transformed *E. coli* colonies by induction of the lac promoter with 0.2mM IPTG (isopropyl β-d-thio galactoside) in log phase cultures grown at 37°C. Cultures were induced at a A_{600} of 1.5 - 2 and incubated for a further 16 hours at 18°C. The cell pellet was then harvested by centrifugation and the contents of the cell periplasm isolated using the protocol of Minsky et al. (1986).

Analysis of the periplasmic fraction by polyacrylamide gel electrophoresis and Western blot probed with mouse anti FLAG® M2 antibody revealed the presence of a FLAG-tagged component in the periplasm with an approximate molecular weight (Mr) of 20 kD. The periplasmic fraction was then assayed by ELISA to reveal the presence of protein molecules with the following properties:

1. The ability to bind to polyclonal anti human IgM antibody prepared in sheep and immobilised on the ELISA.
2. The ability to bind to intact mouse IgG as detected by the addition of goat anti mouse IgG antibody, labelled with horseradish peroxidase which reacts with TMB (3',3',5',5',-tetramethylbenzidine) to produce a coloured product measured at 450 nm.

Property 1 was demonstrated by an ELISA in which crude periplasm was reacted with immobilised polyclonal anti human IgM capture antibody, then probed with mouse anti FLAG® antibody together with goat anti mouse IgG labelled with horseradish peroxidase to detect the C terminal FLAG tag. ELISA plate wells were coated with polyclonal sheep anti human IgM

antiserum (Sang et al., 1998), blocked with 5% Skim milk powder in PBS at 37°C for 1.5 hours. Between each addition step, the wells were washed 10 times with PBS-0.05% Tween 20. Each addition (100 µl) was incubated for 20 min at room temperature. Mouse anti FLAG® (Eastman Kodak Co. New Haven, CT) was used at 1ug/ml in PBS-0.05% Tween 20. Goat anti mouse IgG Fc-HRP was used at 0.16 ug/ml in PBS-0.05% Tween 20. Colour was developed by the addition of 100 µl TMB reagent (3',3',5',5',-tetramethylbenzidine plus H_2O_2), incubation at room temperature for 10 mins followed by the addition of 100 µl 1M Phosphoric acid, and incubation at room temperature for 10 mins. Wells were then read at 450 nm in an ELISA micro plate reader. The results are shown in Table 2.

Table 2

1st addition	2nd addition	3rd addition	A450
Periplasm	mouse anti FLAG®	Goat anti-mouse Ig HRP	>3.000 (4 wells)
PBS	mouse anti FLAG®	Goat anti-mouse Ig HRP	0.124 ± 0.008 (3 wells)
PBS	PBS	Goat anti-mouse Ig HRP	0.094

The positive result could arise from a combination of the binding of anti FLAG via the FLAG epitope, or the binding of the mouse IgG with the FB domain on the bifunctional molecule. Regardless of the proportional contributions from either of these reactions, the result demonstrates that the bifunctional molecule can be captured by anti human IgM capture antibodies.

Property 2 was tested using an ELISA sandwich as shown in Figure 9. Four mouse IgG subclasses were each individually tested for their ability to bind to the bifunctional molecule. Reagents were from AMRAD, Melbourne, Australia (Mouse IgG1: 12CONT01 batch WD12A; IgG2a: 12CONT02 batch UI17A; IgG2b: 12HHLA01 batch UK18A; IgG3 Rota Ser4 batch UK07-B1). Each was diluted to 1 µg/ml with PBS/0.05% Tween20 before use. The control linker reagent FB-$C_H3\mu$ was diluted serially from 1/20 to 1/320 and 100µl loaded into ELISA wells coated with stabilised sheep anti-human IgM

(PanBio Pty Ltd) and incubated for 1 hour at 37°C. After 6 washes with PBS/0.05% Tween20 100µl of each diluted mouse IgG subclass was added and the plate incubated a further hour at 37°C. After 6 washes in PBS/0.05% Tween20, 100µl HRP-labelled goat anti mouse IgG Fc (Pierce Chemical Co. Rockford, Ill) was added at 0.16µg/ml. the plate then incubated for 1 hour and washed 6 times. The reaction was developed with 100µl TMB solution for 10 min. stopped by adding 100 µl 1M phosphoric acid and the absorbance read at 450nm. The results are shown in Table 3.

Table 3

Dilution of Control linker reagent FBCH3µ	Mouse IgG1 1 µg/ml	Mouse IgG 2a 1 µg/ml	Mouse IgG 2b 1 µg/ml	Mouse IgG3 1 µg/ml
1/20	2.425	1.134	0.762	0.599
1/40	2.007	0.555	0.787	0.489
1/80	2.010	0.776	0.578	0.289
1/160	1.429	0.581	0.399	0.373
1/320	1.123	0.320	0.309	0.302
PBS	0.260 (av of 3)			

These results show that under the conditions of the reaction. the binding of mouse IgG subclasses to the control linker reagent is ranked in the following order: IgG1, IgG2a, IgG2b, IgG3, from highest to lowest. Control reagents formed using a Staphylococcus protein A fragment B-C domain linker would be most successful if mouse IgG1 is used to form the complex. It will be appreciated by those skilled in the art that if the subclass of the mouse monoclonal antibody is IgG3, a front end domain other than protein A would preferably be used to produce the bifunctional molecule. Suitable alternatives are described in the "Summary of the Invention" section of this specification..

The bifunctional molecule was separated from other periplasmic components by affinity chromatography on matrix bound mouse anti FLAG® antibody. The fraction which bound to the column was eluted with 0.1 M Glycine HCl pH 3.0 then adjusted to neutrality with saturated Tris.

The bifunctional molecule (denoted FB-$C_H3\mu$) was concentrated to a final concentration of approx 1.2 mg/ml and used in an indirect ELISA test.

Human Herpes Virus 6 (HHV6) Indirect ELISA

Tissue culture supernatant containing mouse monoclonal antibody to HHV6 was diluted 1/50 in PBS-0.05%Tween 20 and added to ELISA plates previously coated with HHV6 antigen and incubated at 37°C for 30 min. After 4 washes with PBS-Tween, FB-$C_H3\mu$ was added to subsequent wells in doubling dilutions from 1/20 to 1/1280 in similar diluent and incubated a further 30 mins at 37°C. After 4 washes with diluent, polyclonal sheep anti human IgM labelled with horseradish peroxidase (AMRAD, Melbourne, 1/1500) was added and incubated 20 min at 37°C. Wells were washed 6 times with PBS and the peroxidase reaction was developed using 100 µl TMB solution (3', 3', 5', 5'-Tetramethylbenzidine; BioChem ImmunoSystems Italia SPA) for 10 mins and the reaction stopped by the addition of 100 µl 1M Phosphoric acid. Results are presented in Table 4. The results demonstrate an effective positive reaction to dilutions as great as 1/80.

Table 4

Dilution of FB-$C_H3\mu$	A_{450}
No FB-$C_H3\mu$ (zero)	0.050
1/20	0.771
1/40	0.512
1/80	0.384
1/160	0.197
1/320	0.139
1/640	0.087
1/1280	0.079

Use of FB-$C_H3\mu$ control linker in AMRAD Hepatitis E antibody indirect ELISA

The control linker FB-$C_H3\mu$ was mixed with mouse IgG1 monoclonal antibody to the conformational epitope of Hepatitis E virus (Ref code 2E2) and used in an indirect ELISA test, comparing the response to positive and negative serum controls provided with the test kit (AMRAD, Melbourne, Vic).

The control linker sample was partially purified and concentrated from material located in the periplasmic fraction. The control linker and mouse HEV antibody were mixed prior to the assay such that there was a dilution series of mouse monoclonal antibody from 0 to 50 µg/ml at control reagent dilutions of 1:10 and 1:50. The human positive control was serially diluted from 1/200 and the negative control diluted 1/200 with serum diluent supplied with the kit. Samples were added to an ELISA plate (AMRAD hepatitis E virus coated plates batch #1401H037) and incubated at room temperature for 30 min. After 3 washes with PBS/0.05% Tween20. 100µl anti-human IgM–HRP conjugate (Silenus; 1:10,000) was added. incubated a further 30 min at room temperature, washed 3 times and TMB substrate added. After 10 min incubation, the reaction was stopped with 1M sulphuric acid and the plate read at 450nm. The results are shown in Table 5.

Table 5

2E2 Mab conc (µg/ml)	Control reagent Dilution		Positive Control Dilution Series	
	1:10	1:50	Dilution	A450
50	2.92	1.828	1/200	2.745
25	2.931	1.795	1/400	2.135
10	2.880	1.772	1/800	1.525
5	2.900	1.703	1/1600	0.799
2	2.378	1.312	1/3200	0.505
1	2.112	0.792	1/6400	0.296
0	0.025	0.023	1/12800	0.168
			Negative Control 1/200	0.103

These results show that Premixed FB-$C_H3\mu$ control linker/ mouse monoclonal antibody can serve as a suitable positive IgM control in the AMRAD HEV ELISA assay.

Levels which give comparable A450 to serum controls are:

Control Linker 1/10 + Mab 2 µg/ml

Control Linker 1/50 + Mab 50 µg/ml.

No significant background problems are observed indicating that this is a viable option to serum controls in the HEV assay.

EXAMPLE 5

Production of a bifunctional molecule containing the B fragment of Staphylococcal Protein A linked to a human IgG C-domain

The gene sequences of human IgG constant domains 2 and 3 were separately amplified from cDNA prepared from mRNA isolated from human peripheral blood lymphocytes using polymerase chain reaction techniques. The design of the oligonucleotide primers used in the amplifications was based upon the 5' and 3' sequences for each of the heavy chain exons, obtained through Genbank accession no E06998.

Whereas *Staphylococcal* protein A (SPA) exhibits a stronger affinity for human IgG1, 2 and 4 than for mouse IgG subclasses. binding to human IgG3 is negligible (Reis et al,1984). It has been suggested that the substitution of histidine with arginine at position 435 in IgG3 prevents the binding to Protein A (Deisenhofer, 1981). Therefore in order to minimise any self aggregation of a bifunctional construct between Fragment B of SPA and human IgG C domains, it would be preferable to have any C3γ domain sequence contain the IgG3 mutation, Arg^{435}. It is not possible to selectively amplify IgG3 constant region sequences from cDNA because of the close homology of the 5' and 3' terminal sequences between all human IgG subclasses. Consequently the mutation was performed subsequent to the amplification and cloning using the QuikChange™ Site Directed mutagenesis kit (Stratagene, La Jolla, CA).

Sequences coding for *Not*I and *Sac*II sites were added to the 5' and 3' end respectively of $C_H2\gamma$ and $C_H3\gamma$ sequences to enable insertion into the expression vector pGC FB-$C_H3\mu$, shown in Figure 8, from which the $C_H3\mu$ sequence was removed as a *Not*I—*Sac*II fragment.

Expression in *E. coli* and purification of product was performed as described in Example 4.

PanBio IgG Indirect Dengue ELISA using FB-$C_H2\gamma$ and FB-$C_H3\gamma$ control linkers

Test samples were unfractionated periplasmic fractions containing FB-$C_H2\gamma$ and FB-$C_H3\gamma$ control linkers from 500 ml expression cultures. Samples were used neat or diluted 1:10 in PBS/0.05% Tween 20.

Mouse anti dengue monoclonal antibody was clone 13C11 (IgG2a) obtained from PanBio Ltd (Windsor, Qld) at 1.6 mg/ml and used at a final concentration of 1.6 µg/ml diluted in PBS/0.05% Tween 20.

The human positive serum control containing anti dengue IgG antibodies was obtained from PanBio Ltd and is identical to what is supplied in their commercial dengue ELISA test. It was used at a dilution of 1:100 in PBS/0.05% Tween 20.

HRP-labelled sheep anti human IgG (lot TJ19B) was from Silenus/AMRAD (Melbourne) and used at a dilution of 1:1000 in PBS/0.05% Tween 20.

The ELISA plate coated with dengue antigens was as supplied by PanBio in their commercial Dengue ELISA test. It was used without further blocking. All incubations were for 1 hour at 37°C followed by 3x2min washes with PBS/0.05% Tween 20.

The first layer of the ELISA contained 100 µl 13C11 mouse anti dengue monoclonal antibody; control wells contained PBS/0.05% Tween 20. Following incubation and washing as described the samples containing FB-$C_H2\gamma$ and FB-$C_H3\gamma$ were added. Controls contained either human anti dengue IgG serum 1:100 or PBS/0.05% Tween 20. Following incubation and washing, HRP-labelled sheep anti human IgG 1:1000 was added. After incubation and washing, 100 µl TMB solution (containing H_2O_2) was added and incubated for 10 min at room temperature to develop the colour reaction. 100µl 1M phosphoric acid was added to stop the reaction and the plate read in a micro plate reader at 450nm. Results are shown in Table 6.

Table 6

	A450	
13C11mAb + FB- $C_H3\gamma$ periplasm	1.475	1.220
PBS-Tween + FB-$C_H3\gamma$ periplasm (control)	0.564	
13C11mAb + FB-$C_H3\gamma$ periplasm 1:10	0.901	0.825
PBS-Tween + FB-$C_H3\gamma$ periplasm 1:10 (control)	0.268	
13C11mAb + FB-$C_H2\gamma$ periplasm	0.856	0.814
PBS-Tween + FB-$C_H2\gamma$ periplasm (control)	0.411	
13C11mAb + FB-$C_H2\gamma$ periplasm 1:10	0.545	0.521
PBS-Tween + FB-$C_H2\gamma$ periplasm 1:10 (control)	0.279	
PBS-Tween + human anti dengue IgG positive control 1:100	0.930	0.922
PBS-Tween + PBS-Tween	0.276	0.265

Both periplasmic fractions containing FB-$C_H2\gamma$ and FB-$C_H3\gamma$ provide positive reactions in this ELISA when linked with the mouse anti dengue mAb, 13C11, compared to controls. The linker containing the $C_H3\gamma$ domain is the preferred construct to mix with a specific mouse monoclonal antibody to use as a replacement IgG positive control reagent.

EXAMPLE 6

Bifunctional construct using core streptavidin as the Ig binding domain

The protein streptavidin produced by *Streptomyces sp.* has an affinity (K_D) for biotin of the order of 10^{-15} M (Green, 1975; Pähler et al., 1987) Commercially produced streptavidin consists of a N- and C- terminally shortened form, called core streptavidin (Argaraña et al., 1986) comprising the sequence from Ala^{13} or Glu^{14} to Ala^{138} to Ser^{139} of the mature polypeptide. Core streptavidin is more soluble than the full length protein and its binding activity for biotinylated proteins is significantly enhanced (Bayer et al., 1989).

The nucleotide sequence for the intact streptavidin gene from *Streptomyces avidinii* was obtained from Genbank accession no. X03591 (Argaraña et al., 1986).

The structural gene encoding core streptavidin was amplified from chromosomal DNA of *S. avidinii* (ATCC27419) using *Pfu* DNA polymerase and oligonucleotides able to recognise the 5´ and 3´ sequences of the core streptavidin (codons from Ala^{13} to Ser^{139}). The oligonucleotide primers also contained sequences flanking the 5´ and 3´ streptavidin sequences for restriction sites (in particular *Nco*I at the 5´end and *Not*I at the 3´ end to enable the core streptavidin gene to be inserted into the vector pGC (Coia et al., 1996) which already contains the sequence for the human IgM C_H3 domain. in the configuration streptavidin-C domain. The sequence coding for the FLAG® tag epitope (Hopp et al., 1998) lies 3´ to the C domain to enable the FLAG® tag to be expressed as a C-terminal peptide on the molecule.

The amplified core streptavidin gene was inserted into the PCR-Script™ SK(+) plasmid using the PCR-Script™ Cloning Kit obtained from Stratagene, La Jolla. CA (Cat no. 211190-5). After the DNA sequence was confirmed in positive transformants, the core streptavidin sequence was excised from the plasmid by double digestion with *Nco*I and *Not*I. and ligated into a likewise digested pGC vector containing the DNA sequence for human IgM C_H3 domain.

The verified sequence of the expression cassette in pGC comprising the pel B leader sequence, core streptavidin, human IgM C_H3 domain and FLAG® tag is shown in SEQ ID NO: 4 and Figure 10.

Expression in *E. coli* was performed as described in Example 4. Cells from a 500 ml culture were fractionated into periplasmic fraction, cytoplasmic and membrane fraction. The periplasmic fraction was prepared using the protocol of Minsky et al. (1986). The cell pellet remaining after centrifugation to obtain the periplasmic supernatant was resuspended in TE buffer (10mM Tris HCl pH 7.4, 1mM EDTA) sonicated and centrifuged at 20,000xg to obtain the soluble cytoplasmic fraction and the membrane pellet. Western blot analysis of each of the three fractions using the FLAG® tag as a probe indicated that while the expressed product was present in all three fractions, the membrane pellet contained the highest levels.

The membrane fraction was dissolved in 10 ml 6M guanidinium HCl. pH 1.5 (Schmidt and Skerra, 1994), dialysed twice against 200ml 6M guanidinium HCl, pH 1.5, then twice against 2L PBS at 4°C. After centrifugation to remove insoluble aggregate, the supernatant was fractionated on a Superdex 200 (HR 10/30, Pharmacia LKB Biotechnology)

column run in PBS at 0.5ml/min. The elution profile is shown in Figure 11. All three peaks probed with FLAG® indicating the presence of the product. The first peak is high molecular weight aggregate eluting at the void volume of the column. The second and third peaks were collected separately and labelled preparation B (0.10 mg/ml)and preparation A (0.17 mg/ml) respectively. Both preparations showed bands on Western blot of identical size, and so the size difference between the two preparations is related to the multimerisation state of the product. Both preparations were used in tests as described below.

Use of Control Reagent Streptavidin-$C_H3\mu$ in PanBio Dengue Indirect ELISA

In this test, a complex is formed between biotinylated monoclonal mouse IgG to dengue antigens (13C11-B) and streptavidin linked to human IgM C_H3 domain (strep-$C_H3\mu$) to mimic positive human IgM antibody to dengue, and used as a pseudo positive control in commercial IgM capture Dengue ELISA and indirect IgM Dengue ELISA kits where the response is compared to positive and negative controls provided in the kit.

Biotinylation of mouse monoclonal anti dengue IgG (Clone 13C11)

2.56 mg of 13C11 Monoclonal antibody (IgG fraction) to dengue antigen (PanBio Ltd, Windsor, Qld; product 13C7001) was equilibrated in 1ml 50mM sodium bicarbonate buffer, pH 8.0. To this was added 75µl freshly prepared EZ-Link™ Sulfo-NHS-LC-Biotin solution (1mg/ml in water) (Pierce Chemical Company, Rockford, IL; product code 21335), and incubated at room temperature for 1 hour. After the sample had undergone extensive dialysis against PBS (phosphate buffered saline, final sample volume 1.2ml), the protein concentration was estimated by absorbance at 280nm to be 1.6 mg/ml.

The success of the biotinylation reaction was confirmed by ELISA, in which dilutions of the biotinylated 13C11 antibody was added to wells containing immobilised dengue antigen. Non biotinylated 13C11 was used as a negative control. A streptavidin-horse radish peroxidase conjugate was used to visualise the presence of the biotinylated 13C11 antibody.

Dengue IgM Indirect ELISA

The reagents used in this assay were as follows:

- ELISA plate coated with dengue 2 antigen (PanBio Pty Ltd, Windsor Qld)

- IgM Positive Control Serum, IgM Cut-off Calibrator Serum. Negative Control Serum for IgM (PanBio Pty Ltd provided in the kit)
- Biotinylated mouse anti dengue IgG (clone 13C11), concentration = 1.6 mg/ml (biotinylation was performed as described above).
- Non biotinylated mouse anti dengue IgG (clone 13C11), concentration = 1.6 mg/ml
- Control Linker Reagent: Strep-$C_{H}3\mu$ Preparation A, concentration 0.17mg/ml.

Control linker reagent was mixed with biotinylated and non-biotinylated 13C11 Mab prior to the assay in the following proportions:

1. 13C11-Biotin (1/10,000) + Control Linker Prep A 1/10
2. 13C11-Biotin (1/10.000) + Control Linker Prep A 1/100
3. 13C11-Biotin (1/10,000) + Control Linker Prep A 1/1000
4. 13C11 (1/10,000) [nonbiotinylated] + Control Linker Prep A 1/10
5. 13C11 (1/10,000) [nonbiotinylated] + Control Linker Prep A 1/100
6. 13C11 (1/10,000) [nonbiotinylated] + Control Linker Prep A 1/1000

Dilution was in serum diluent (Tris buffered saline with preservatives and additives) as supplied in the kit. Mixing took place at room temperature for 10 min with rotation.

Additions to ELISA plate were in the following order:

1. Samples as above, blank, positive, negative and cut-off serum controls (100µl) incubated at 37°C for 1 hour, followed by 6 washes with diluted wash buffer.
2. HRP-labelled sheep anti Human IgM (Silenus/AMRAD; Code MAH) 100µl of 1/1000 dilution; incubated for 1 hour at 37°C followed by 6 washes with diluted wash buffer.
3. Reaction was developed with 100 µl TMB reagent 10 min at room temperature, followed by the addition of 100 µl 1M phosphoric acid. Samples were read at 450nm.

The results obtained from this indirect assay are shown in Table 7.

Table 7

Sample	A450
Blank (Serum diluent only)	0.058
Negative serum Control 1/100	0.063
Positive Serum Control 1/100	1.419
Positive Cut-Off 1/100	0.522
Positive Cut-Off 1/100 (duplicate)	0.580
Control Linker 1/10 + 13C11-biotin	1.186
Control Linker 1/10 + 13C11-biotin (dup)	1.109
Control Linker 1/100 + 13C11-biotin	1.070
Control Linker 1/100 + 13C11-biotin (dup)	1.076
Control Linker 1/1000 + 13C11-biotin	0.212
Control Linker 1/1000 + 13C11-biotin (dup)	0.183
Control Linker 1/10 + 13C11	0.151
Control Linker 1/100 + 13C11	0.059
Control Linker 1/1000 + 13C11	0.074

These results show tha the complex formed between the Strep-$C_H3\mu$ linker reagent and the biotinylated mouse anti dengue IgG (13C11) acted as a human positive control up to a dilution of at least 1/100. No reaction was observed if the mouse monoclonal IgG was not biotinylated, or if there was insufficient control linker reagent to capture the biotinylated mouse Mab.

Use of Control Reagent Streptavidin-$C_H3\mu$ in PanBio Dengue IgM Capture ELISA

The Dengue IgM capture ELISA test kit from PanBio Ltd (Windsor, Qld; Cat No DEMF-200) was used for this demonstration. The positive and cut-off control sera provided contain human IgM antibodies to dengue. The negative control serum contains human IgM antibodies, but with no specificity for dengue antigens.

Two test samples of the Streptavidin-Human C_H3 μ domain linker reagent were used:

a) Preparation A, 0.17 mg/ml

b) Preparation B. 0.10 mg/ml

These preparations were mixed with biotinylated and non-biotinylated 13C11 Mab as described below.

The following samples were prepared for ELISA:

1. Blank – 100 µl serum diluent only, provided in PanBio kit
2. Negative Control Serum–from PanBio kit; 100 µl. diluted 1/100 in serum diluent.
3. IgM Positive Control Serum–from PanBio kit; 100 µl, diluted 1/100 in serum diluent.
4. Cut-off Calibrator–from PanBio kit: 100 µl. diluted 1/100 in serum diluent.
5. Strep-$C_H3\mu$ Preparation A + 13C11-Biotin: 10µl of prepA diluted to 1ml with serum diluent to which 1µl 13C11-Biotin (1/1000 dilution) was added (Final concentration of PrepA protein = 1.7 µg/ml; 13C11-Biotin = 1.6 µg/ml).
6. Strep-$C_H3\mu$ Preparation B + 13C11-Biotin: 10µl of prepB diluted to 1ml with serum diluent to which 1µl 13C11-Biotin (1/1000 dilution) was added (Final concentration of PrepB protein = 1.0 µg/ml; 13C11-Biotin = 1.6 µg/ml).
7. Blank + 13C11-Biotin (negative control) 1µl 13C11-Biotin was added to 1ml serum diluent (Final concentration of 13C11 biotin = 1.6 µg/ml).
8. Strep-$C_H3\mu$ Preparation A + 13C11(non biotinylated) (negative control): 10µl of prepA diluted to 1ml with serum diluent to which 1µl 13C11 (1/1000 dilution) was added (Final concentration of PrepA protein = 1.7 µg/ml; 13C11 = 1.6 µg/ml).
9. Strep-$C_H3\mu$ Preparation B + 13C11(non biotinylated) (negative control): 10µl of prepB diluted to 1ml with serum diluent to which 1µl 13C11 (1/1000 dilution) was added (Final concentration of PrepB protein = 1.0 µg/ml; 13C11 = 1.6 µg/ml).
10. Blank + 13C11 (negative control) 1µl 13C11-Biotin was added to 1ml serum diluent (Final concentration of 13C11 biotin = 1.6 µg/ml).

Each sample was mixed on a rotating wheel for 10 min at room temperature, then 100 µl of each (some in duplicate) were added to ELISA strips from the test kit which were pre-coated with polyclonal sheep anti human IgM. The strips were covered and incubated at 37°C for 60 min, then washed three times for 2 min with PBS containing 0.05% Tween 20. At the same time as the above incubation, 2ml conjugated monoclonal antibody tracer (PanBio: anti dengue–HRP) was added to one vial of

lyophilised dengue antigen (serogroups 1-4) and rocked gently at room temperature to aid in the dissolution of the dengue antigen. After the above washes, 100µl of the HRP conjugate was added to each well, incubated for 60 min at 37°C then washed three times for 2 min with PBS-0.05% Tween 20. 100µl of TMB reagent (3´,3´,5´.5´ tetramethylbenzidine/hydrogen peroxide; supplied with the kit) was then added to each well and the strips incubated at room temperature for 10 mins. The reaction was stopped by the addition of 100µl 1M phosphoric acid and the colour intensity read at 450 nm. Results of this assay are shown in Table 8.

Table 8

Sample	A450
Blank (serum diluent only)	0.115
Negative serum control	0.121
IgM positive serum control	2.678
Positive Cut-off Calibrator	1.198
Positive Cut-off Calibrator (duplicate)	1.235
Preparation A + 13C11-biotin	1.914
Preparation A + 13C11-biotin (duplicate)	1.850
Preparation B + 13C11-biotin	1.200
Preparation B + 13C11-biotin (duplicate)	1.344
Blank + 13C11-biotin	0.115
Blank + 13C11-biotin (duplicate)	0.128
Preparation A + 13C11(non biotinylated)	0.142
Preparation A + 13C11(non biotinylated)	0.126
Preparation B + 13C11(non biotinylated)	0.113
Preparation B + 13C11(non biotinylated)	0.124
Blank + 13C11(non biotinylated)	0.106

Both preparations of Strep-$C_H3\mu$ gave positive reactions in the ELISA at levels sufficient for the complex with mouse IgG to be used as a replacement for the positive control serum. The lower reading with preparation B can be partly attributed to the lower concentration of the product. Results with the controls indicated that there were no significant background problems.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References:

Åkerström. B. and Björck, L (1989) Protein L: an immunoglobulin light chain-binding bacterial protein. Characterization of binding and physicochemical properties. J. Biol. Chem. 264: 19740-19746.

Argaraña, C.E., Kuntz, I.D., Birken. S., Axel. R. and Cantor, C.R. Molecular cloning and nucleotide sequence of the streptavidin gene. Nucl. Acids Res. 14 1871-1882.

Bayer, E.A., Ben-Hur, H., Hiller, Y., and Wilchek, M. (1989) Post-secretory modifications of streptavidin. Biochem. J. 259 369-376.

Better, M., Chang, C.P., Robinson, R.R., and Horwitz, A.H. (1988) Escherichia coli secretion of an active chimeric antibody fragment. Science 240, 1041-1043.

Björck, L. and Kronvall, G. (1984) Purification and some proerties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol. 133, 969-974.

Borza, D-B., Tatum, F.M. and Morgan, W.T. (1996) Domain Structure and Conformation of Histidine-Proline-Rich Glycoprotein. Biochemistry 35 1925-1934.

Boulianne, G.L., Hozumi, N. and Shulman, M.J. (1984) Production of functional chimaeric mouse/human antibody. Nature (Lond.) 312, 643-646.

Boyle, M.D.P. and Reis, K.J. (1987) Bacterial Fc receptors. Bio/technology 5, 697-703.

Coia G., Hudson, P. J. and Lilley G. G. (1996) Construction of recombinant extended single-chain antibody peptide conjugates for use in the diagnosis of HIV1 and HIV2. J. Immunol. Methods. 192, 13-23.

Diesenhofer, J., (1981). Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8 Å resolution. Biochemistry 20, 2361-2370.

Dolezal, O., Coia, G., Guthrie, R.E., Lilley, G.G. and Hudson, P.J. (1995) Escherichia coli expression of a bifunctional Fab-peptide epitope reagent for the rapid diagnosis of HIV-1 and HIV-2 Immunotechnology 1 197-209.

Dorai, H. and Gillies, S.D. (1989) The complete nucleotide sequence of a human immunoglogulin genomic C μ gene. Nucleic Acids Research 17, 6412

Gorgani, N.N., Parish, C.R., Easterbrook Smith, S.B., and Altin, J.G. (1997) Histidine-Rich Glycoprotein binds to Human IgG and C1q and inhibits the formation of insoluble immune complexes. Biochemistry 36 6653-6662.

Green, N.M. (1990) Avidin and Streptavidin. Methods in Enzymol. 184 51-67.

Hamilton. R.G. (1990) Engineered human antibodies as immunologic quality control reagents. Ann. Biol. Clin. 48, 473-477.

Hamilton R.G. (1991) Application of engineered chimeric antibodies to the calibration of human antibody standards. Ann. Biol. Clin. 49, 242-248.

Heusser C.H., Anderson, C.L. and Grey, H.M. (1977) Receptors for IgG: subclass specificity of receptors on different mouse cell types and the definition of two distinct receptors on a macrophage cell line. J. Exp. Med 145, 1316-1327.

Hogarth, P.M., Hibbs, M.L., Bonadonna, L., Scott, B.M., Witort. E. Pieterz, G.A. and McKenzie, I.F. (1987) Immunogenetics 26, 161-168.

Hopp, T. P., Prickett. K. S., Price, V.L., Libby, R. T., March C. J., Cerretti, D. P., Urdal, D. L. and Conlon, P. J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. Bio/Technology 6, 1204-1210.

Huston, J. S., Mudgett-Hunter, M., Tai,M.-S., McCartney, J. E., Warren, F. D., Haber, E., and Oppermann. H (1991) Protein engineering of single chain Fv analogs and fusion proteins. Methods Enzymol. 203, 46-88.

Kulcczycki, A. Jr., Webber, J., Soares, H.A., Onken. M.D., Thompson, J.A., Chaplin. D.D., Loh, D.Y. and Tillinghast J.P. (1990) Genomic organization of mouse Fc receptor genes. Proc. Natl. Acad. Sci. USA 87, 2856-2960.

Minsky, A., Summers, R.G. and Knowles, J.R. (1986) Secretion of beta lactamase into the periplasm of Escherichia coli: Evidence for a distinct release step associted with a conformational change. Proc. Nat.l Acad. Sci. USA 83 4180-4184.

Morrison, S.L., Johnson, M.J. Herzenberg, L.A. and Oi, V.T. (1984) Chimeric human antibodies: mouse antigen binding domains with human constant regions. Proc Natl. Acad. Sci. USA 81, 6851-6855.

Pähler, A., Hendrickson, W.A., Kolks, M.A., Aragaña, C.E., and Cantor C.R. Characterization and crystallization of core streptavidin J. Biol. Chem. 262 13933-13937.

Reichmann, L., Clark, M., Waldmann, H. and Winter G. (1998) Reshaping human antibodies for therapy. Nature (Lond.) 332, 323-327.

Reis, K.J., Ayoub, E.M., and Boyle, M.D.P. (1984) Streptococcal Fc Receptors. II Comparison of the reactivity of a receptor from a group C Streptococcus with staphylococcal protein A. J. Immunol. 132, 3098-3102.

Rylatt, D. B., Kemp, B. E., Bundesen, P. G., John, M. A., O'Reilly, E. J., Cottis, L. E., Miles, S. J., Khan, J. M., Dinh, D. P., Stapleton, D. and Hillyard, C. J. (1990). A rapid whole-blood immunoassay system. Med. J. Aust. 152, 75-77.

Sang, C.T., Cuzzubbo, A.J. and Devine, P.L. (1998) Evaluation of commercial capture Enzyme -Linked Immunosorbent Assay for the determination of IgM

and IgG antibodies produced during dengue infection. Clinical Diagnostic Laboratory Immunology 5, (1) in press.

Schmidt, T.G.M. and Skerra, A. (1994) One-step affinity purification of bacterially produced proteins by means of the "Strep tag" and immobilized recombinant core streptavidin. J Chromatog. A 676, 337-345.

Segal D.M., and Titus, J.A. (1978) The subclass specificity for the binding of murine myeloma proteins to macrophages and lymphocyte cell lines and to normal spleen cells J. Immunol. 120, 1395-1403.

Skerra A. (1993) Bacterial expression of immunoglobulin fragments. Curr. Opin. Immunol. 5, 256-262.

Uhlen, M., Guss, B., Nilsson, B., Gatenvack, S., Philipson, L and Lindberg, M. (1984) Complete sequence of the Staphylococcal gene encoding Protein A. J. Biol. Chem. 259, 1695-1702.

Unkeless, J.C., Scigliano, E. and Freedman, V.H. (1988) Structure and function of human and murine receptors for IgG (1988) Ann. Rev. Immunol 6, 251-281.

Winter, G. and Milstein, C. (1991) Man-made antibodies. Nature (Lond.) 349, 293-299.

Yanisch-Perron, C., Vieira, J., and Messing, J.(1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

Zhou, H., Fisher, R.J. and Papas, T.S. (1994) Optimization of primer sequences for mouse repertoire display library construction. Nucleic Acids Research 22, 888-889.

Claims:

1. A chimeric antibody conjugate comprising an antigen binding region of a non-human antibody and an immunoglobulin constant region which comprises at least one C_H domain or epitope thereof, with the proviso that the constant region is not a naturally occurring F_C fragment.

2. A chimeric antibody conjugate according to claim 1 in which the non-human antigen binding region comprises or consists of a non-human Fab fragment or part thereof.

3. A chimeric antibody conjugate according to claim 1 or claim 2 in which the non-human antigen binding region comprises or consist of an scFv fragment.

4. A chimeric antibody conjugate according to any one of claims 1 to 3 in which the non-human antigen binding region is derived from a mouse.

5. A chimeric antibody conjugate according to any one of claims 1 to 4 in which the constant region is derived from a human antibody.

6. A chimeric antibody conjugate according to any one of claims 1 to 5 in which the constant region comprises one or more constant domains derived from an IgM antibody.

7. A chimeric antibody conjugate according to claim 6 in which the constant region comprises one or more $C_H3\mu$ domains.

8. A chimeric antibody conjugate according to any one of claims 1 to 5 in which the constant region comprises one or more constant domains derived from an IgG antibody.

9. A chimeric antibody conjugate according to claim 8 in which the constant region comprises one or more $C_H3\gamma$ domains.

10. A chimeric antibody conjugate according to any one of claims 1 to 5 in which the constant region comprises one or more constant domains derived from an IgA antibody.

11. A chimeric antibody conjugate according to any one of claims 1 to 10 in which the constant region comprises a non-naturally occurring combination of C_H domains or epitopes thereof.

12. A chimeric antibody conjugate according to any one of claims 1 to 11 in which the non-human antigen binding region binds to an epitope of an infectious agent selected from dengue virus, rubella virus, herpes virus, parvovirus, human glycophorin, *Rickettsia sibirica, Burkholeria pseudomallei, Salmonella typhi* or *paratyphi, Leptospira interrogans, Plasmodium falciparum/vivax,* Japanese encephalitis virus, Yellow fever virus, *Bordetella pertussis/parapertussis, Candida albicans/kruzei,* Varicella zoster virus, HIV, Hepatitis viruses, Human papilloma virus, Epstein-Barr virus, Ross River virus, *Brucella abortis,* Human herpesvirus-6, Parvovirus B19, *Coxiella burnettii,* Herpes simplex viruses 1&2, *Rickettsia rickettsii, Conori australis,* and *Rickettsia tsutsugamushi.*

13. A recombinant polynucleotide molecule comprising a sequence encoding a non-human V_H region, a sequence encoding a non-human V_L region, a sequence encoding a flexible linker positioned between the V_H region sequence and the V_L region sequence, and a heterologous sequence encoding a C_H domain or epitope thereof.

14. A recombinant polynucleotide molecule according to claim 13 in which the heterologous sequence encodes a human C_H domain.

15. A recombinant polynucleotide molecule according to claim 13 or claim 14 in which the C_H domain sequence is linked to the 3' end of the V_L or V_H sequence.

16. A recombinant polynucleotide molecule according to any one of claims 13 to 15 in which the polynucleotide molecule includes a control

sequence which directs the synthesis of both the V_L and V_H polypeptide regions.

17. A recombinant polynucleotide molecule according to claim 16 in which the control sequence is the lac promoter.

18. A recombinant polynucleotide molecule according to any one of claims 13 to 17 in which the molecule includes a sequence encoding a leader peptide which directs the synthesised polypeptide chains to the host cell periplasm.

19. A recombinant polynucleotide molecule according to claim 18 in which the leader sequence is the pel B sequence.

20. A recombinant polynucleotide molecule comprising a sequence encoding a non-human V_L region, a sequence encoding a non-human C_L region, a sequence encoding a non-human V_H region, a heterologous sequence encoding a C_H domain or epitope thereof, and optionally a sequence encoding a non-human C_H1 region.

21. A recombinant polynucleotide molecule according to claim 21 in which the heterologous sequence encodes a human C_H domain.

22. A recombinant polynucleotide molecule according to claim 20 or claim 21 in which the V_L and C_L sequences are linked together so that the V_L and C_L regions are expressed as a single polypeptide.

23. A recombinant polynucleotide molecule according to any one of claims 20 to 22 in which the V_H and C_H1 sequences are linked together so that the V_H and C_H1 regions are expressed as a single polypeptide.

24. A recombinant polynucleotide molecule according to any one of claims 20 to 23 in which the polynucleotide molecule includes a control sequence which directs the synthesis of both the V_L-C_L and V_H-C_H1 polypeptide regions.

25. A recombinant polynucleotide molecule according to claim 24 in which the control sequence is the lac promoter.

26. A recombinant polynucleotide molecule according to any one of claims 20 to 25 in which the polynucleotide molecule includes a sequence encoding a leader peptide which directs the synthesised polypeptide chains to the host cell periplasm.

27. A recombinant polynucleotide molecule according to claim 26 in which the leader sequence is the pel B sequence.

28. A recombinant polynucleotide molecule according to any one of claims 20 to 27 in which the heterologous C_H domain sequence is linked to the V_L-C_L sequences or the V_H-C_H1 sequences so that the expressed heterologous C_H domain is attached to the V_L-C_L polypeptide or the V_H-C_H1 polypeptide.

29. A vector comprising a poynucleotide according to any one of claims 13 to 29.

30. A bacterial, yeast, insect or mammalian host cell transformed with a vector according to claim 29.

31. A method of producing a chimeric antibody conjugate which comprises culturing a host cell according to claim 30 under conditions enabling the expression of the conjugate and optionally recovering the conjugate.

32. A chimeric antibody conjugate produced by a method according to claim 31.

33. A method for detecting an antibody in a biological sample which involves comparing the level of detection obtained with the biological sample to the level of detection obtained with a positive control, wherein the positive control comprises a chimeric antibody conjugate according to any one of claims 1 to 12.

34. A method according to claim 33 in which the biological sample is a human biological sample.

35. A method according to claim 33 or claim 34 in which the antibodies to be detected are antibodies characteristic of a disease selected from dengue fever, japanese encephalitis, rubella, spotted fever, herpes infection, parvovirus infections, melioidosis, typhoid, leptospirosis, malaria, yellow fever, whooping cough, systemic candidiasis/thrush, chicken pox, shingles, AIDS, hepatitis, liver cancer, cervical cancer, infectious mononucleosis, nasopharyngeal carcinoma, Ross River fever, brucella, exanthum subitum (sixth disease/roseola infantum), erythema ingfectiosum (fifth disease), Q fever, cold sores, genital herpes, spotted fever and scrub typhus.

36. A method according to any one of claims 33 to 35 in which the antibody is an IgM antibody.

37. A method according to any one of claims 33 to 35 in which the antibody is an IgG antibody.

38. A method according to any one of claims 33 to 35 in which the antibody is an IgA antibody.

39. A bifunctional molecule for use in labelling an antibody derived from a first species, the bifunctional molecule comprising a binding region which binds to the antibody of the first species or to one or more groups provided thereon, and a constant region derived from an antibody of a second species, the constant region comprising at least one C_H domain or an epitope thereof.

40. A bifunctional molecule according to claim 39 in which the binding and constant regions are separated by a linker molecule.

41. A bifunctional molecule according to claim 40 in which the linker molecule is a peptide of between 1 and 20 amino acids in length.

42. A bifunctional molecule according to claim 41 in which the linker molecule is a peptide of between 2 and 5 amino acids in length.

43. A bifunctional molecule according to any one of claims 39 to 42 in which the binding region is not derived from an antibody.

44. A bifunctional molecule according to any one of claims 39 to 43 in which the binding region binds directly to the antibody derived from the first species.

45. A bifunctional molecule according to claim 44 in which the binding region is derived from a protein selected from the group consisting of, *Streptococcal* protein G, *Staphylococcal aureus* protein A and *Peptostreptococcus magnus* protein L.

46. A bifunctional molecule according to claim 45 in which the binding region comprises fragment B of *Staphylococcus aureus* protein A.

47. A bifunctional molecule according to claim 44 in which the binding region comprises a mouse Fc γ receptor or fragment thereof.

48. A bifunctional molecule according to claim 44 in which the binding region comprises a histidine rich glycoprotein.

49. A bifunctional molecule according to any one of claims 39 to 43 in which the binding region binds to one or more groups provided on the antibody of the first species.

50. A bifunctional molecule according to claim 49 in which the group(s) is a biotin molecule and the binding region comprises streptavidin or a fragment thereof.

51. A bifunctional molecule according to any one of claims 39 to 50 in which the antibody constant region is not a naturally occurring Fc fragment.

52. A bifunctional molecule according to any one of claims 39 to 51 in which the constant region comprises one or more constant domains derived from an IgM antibody.

53. A bifunctional molecule according to claim 52 in which the constant region comprises one or more $C_H3\mu$ domains.

54. A bifunctional molecule according to any one of claims 39 to 51 in which the constant region comprises one or more constant domains derived from an IgG antibody.

55. A bifunctional molecule according to claim 54 in which the constant region comprises one or more $C_H3\gamma$ domains.

56. A bifunctional molecule according to any one of claims 39 to 51 in which the constant region comprises one or more constant domains derived from an IgA antibody.

57. A bifunctional molecule according to any one of claims 39 to 56 in which the antibody constant region comprises or consists of a non-naturally occurring combination of immunoglobulin C_H domains or epitopes thereof.

58. A bifunctional molecule according to any one of claims 39 to 56 in which the antibody constant region comprises or consists of a single C_H domain.

59. A bifunctional molecule according to any one of claims 39 to 58 in which the second species is a human.

60. An isolated polynucleotide encoding a bifunctional molecule according to any one of claims 39 to 59.

61. A vector comprising a polynucleotide according to claim 60.

62. A bacterial, yeast, insect or mammalian host cell transformed with a vector according to claim 61.

63. A method of producing a bifunctional molecule which comprises culturing a host cell according to claim 62 under conditions enabling the expression of the bifunctional molecule and optionally recovering the bifunctional molecule.

64. A bifunctional molecule produced by a method according to claim 63.

65. A complex formed between (i) an antibody or biologically active fragment thereof derived from a first species and (ii) a bifunctional molecule, the bifunctional molecule comprising a binding region which binds to the antibody of the first species or to one or more groups provided thereon, and a constant region derived from an antibody of a second species, the constant region comprising at least one C_H domain or an epitope thereof.

66. A complex according to claim 65 in which the binding region has a K_D for the antibody of the first species, or a group provided thereon, of less than 10^{-6} M.

67. A complex according to claim 66 in which the binding region has a K_D for the antibody of the first species, or a group provided thereon, of less than 10^{-8}M.

68. A complex according to any one of claims 65 to 67 in which the bifunctional molecule binds directly to the antibody derived from the first species.

69. A complex according to claim 68 in which the binding region is derived from a protein selected from the group consisting of, *Streptococcal* protein G, *Staphylococcal aureus* protein A and *Peptostreptococcus magnus* protein L.

70. A complex according to claim 69 in which the binding region comprises fragment B of *Staphylococcus aureus* protein A.

71. A complex according to claim 68 in which the binding region comprises a mouse Fc γ receptor or fragment thereof.

72. A complex according to claim 68 in which the binding region comprises a histidine rich glycoprotein.

73. A complex according to any one of claims 65 to 67 in which the binding region binds to one or more groups provided on the antibody of the first species.

74. A complex according to claim 73 in which the group(s) is a biotin molecule and the binding region comprises streptavidin or a fragment thereof.

75. A complex according to any one of claims 65 to 74 in which the constant region comprises one or more constant domains derived from an IgM antibody.

76. A complex according to claim 75 in which the constant region comprises one or more $C_H3\mu$ domains.

77. A complex according to any one of claims 65 to 74 in which the constant region comprises one or more constant domains derived from an IgG antibody.

78. A complex according to claim 77 in which the constant region comprises one or more $C_H3\gamma$ domains.

79. A complex according to any one of claims 65 to 74 in which the constant region comprises one or more constant domains derived from an IgA antibody.

80. A complex according to any one of claims 65 to 79 in which the antibody constant region comprises or consists of a non-naturally occurring combination of immunoglobulin C_H domains or epitopes thereof.

81. A complex according to any one of claims 65 to 79 in which the antibody constant region comprises or consists of a single C_H domain.

82. A complex according to any one of claims 65 to 81 in which the first species is a rat or mouse.

83. A complex according to any one of claims 65 to 82 in which the second species is a human.

84. A method for detecting an antibody in a biological sample which involves comparing the level of detection obtained with the biological sample to the level of detection obtained with a positive control, wherein the positive control comprises a complex according to any one of claims 65 to 83.

85. A method according to claim 84 in which the biological sample is a human biological sample.

86. A method according to claim 84 or claim 85 in which the antibodies to be detected are antibodies characteristic of a disease selected from dengue fever, Japanese encephalitis, rubella, spotted fever, herpes infection, parvovirus infections, melioidosis, typhoid, leptospirosis, malaria, yellow fever, whooping cough, systemic candidiasis/thrush, chicken pox, shingles, AIDS, hepatitis, liver cancer, cervical cancer, infectious mononucleosis, nasopharyngeal carcinoma, Ross River fever, brucella, exanthum subitum (sixth disease/roseola infantum), erythema infectiosum (fifth disease), Q fever, cold sores, genital herpes, spotted fever and scrub typhus.

87. A method according to any one of claims 84 to 86 in which the antibody is an IgM antibody.

88. A method according to any one of claims 84 to 86 in which the antibody is an IgG antibody.

89. A method according to any one of claims 84 to 86 in which the antibody is an IgA antibody.

Figure 1

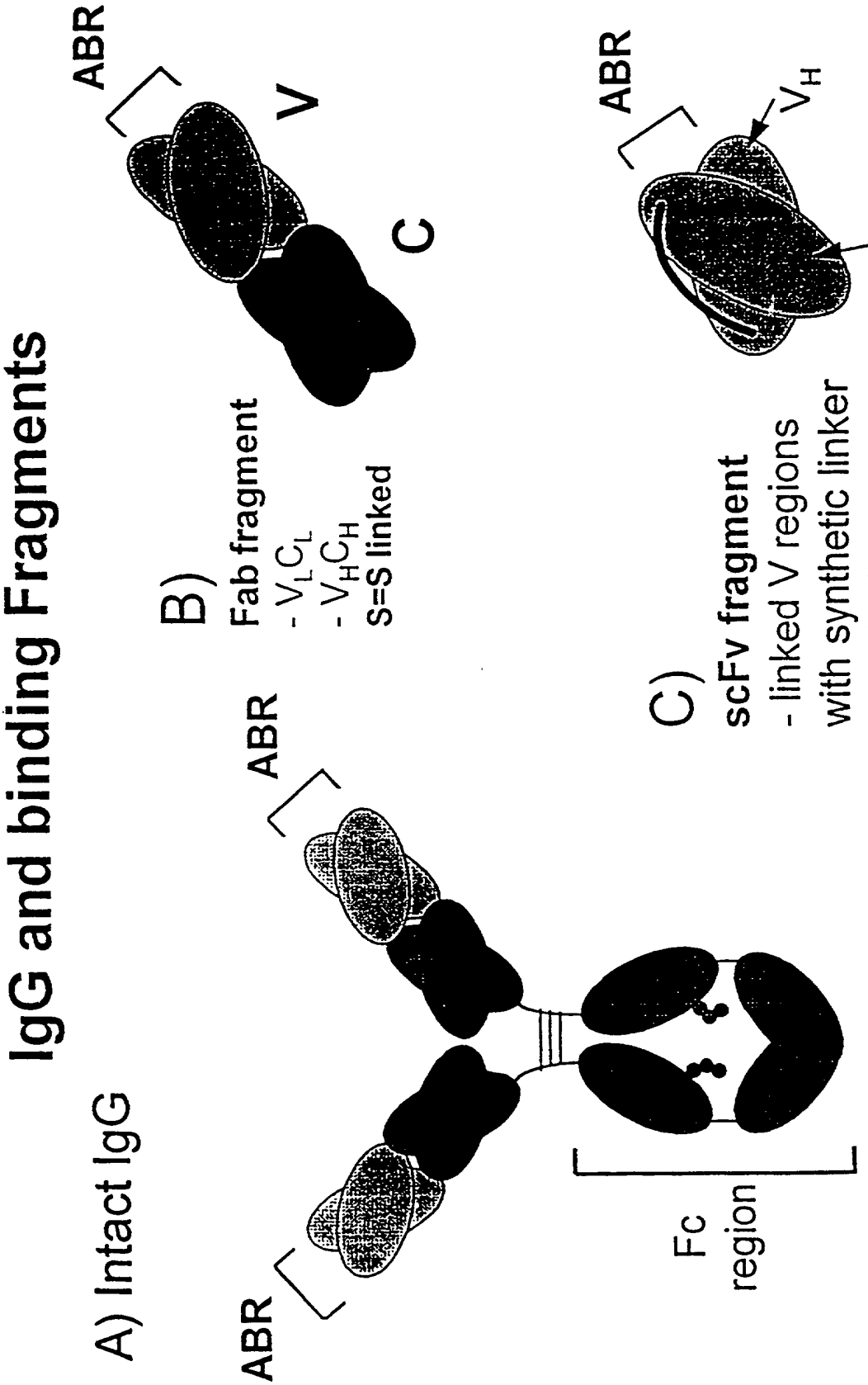

IgG and binding Fragments
A) Intact IgG
ABR
ABR
Fc region
B)
Fab fragment
- V_LC_L
- V_HC_H
S=S linked
ABR
V
C
C)
scFv fragment
- linked V regions
with synthetic linker
$(Gly_4Ser)_3$
ABR
V_H
V_L

Figure 2

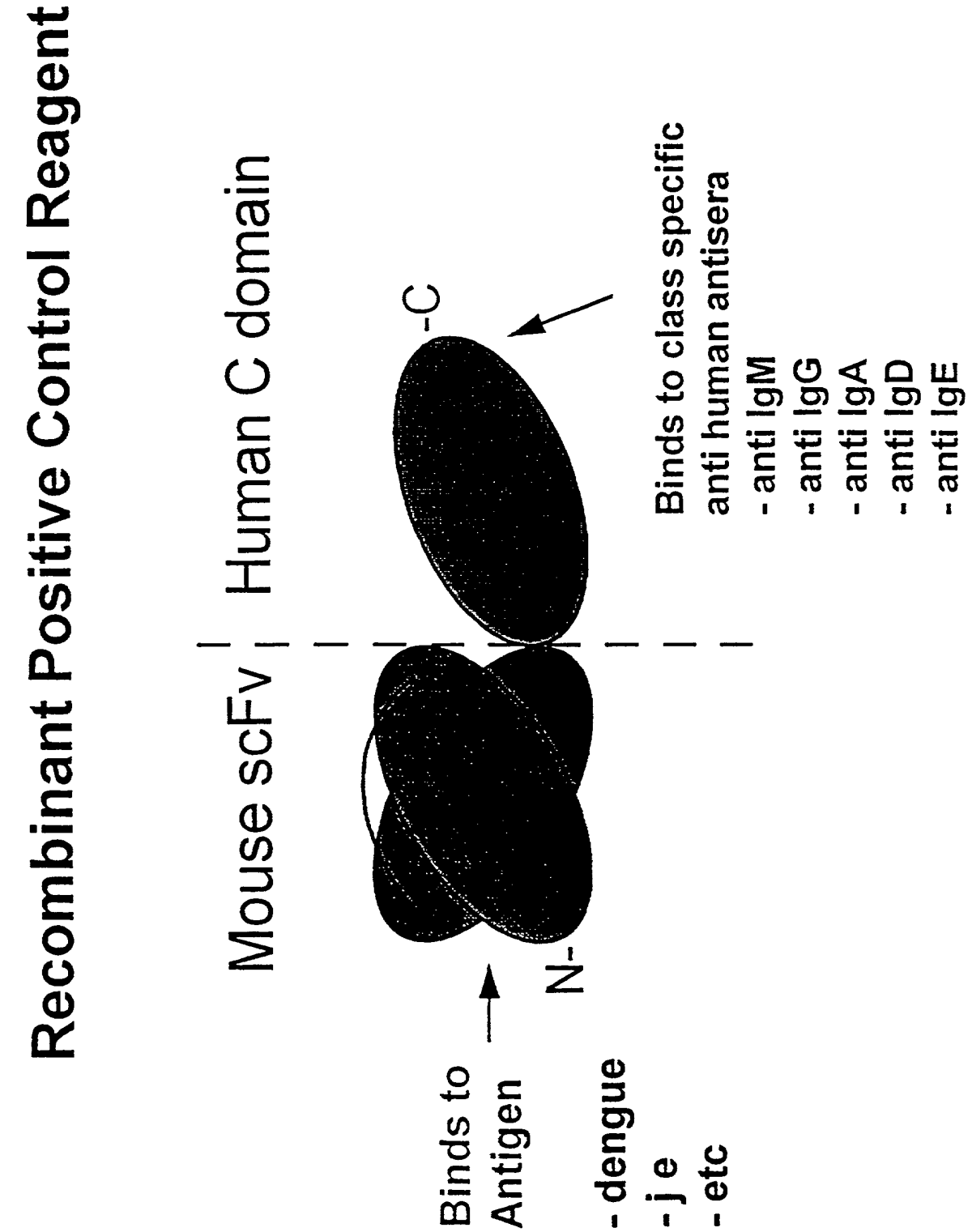
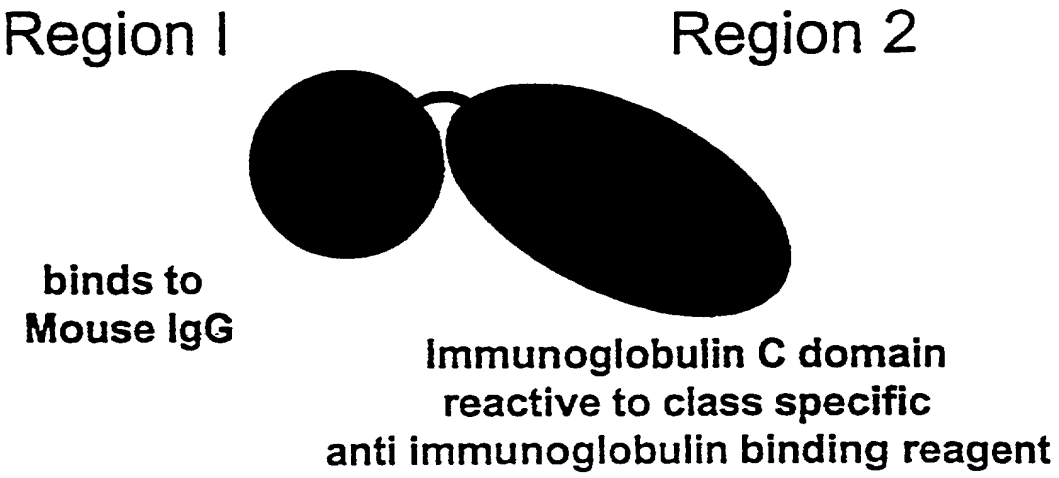

Recombinant Positive Control Reagent
Mouse scFv
Human C domain
-C
N-
Binds to class specific anti human antisera
- anti IgM
- anti IgG
- anti IgA
- anti IgD
- anti IgE
Binds to Antigen
- dengue
- j e
- etc

Figure 3



Complex formed between bifunctional molecule and mouse IgG

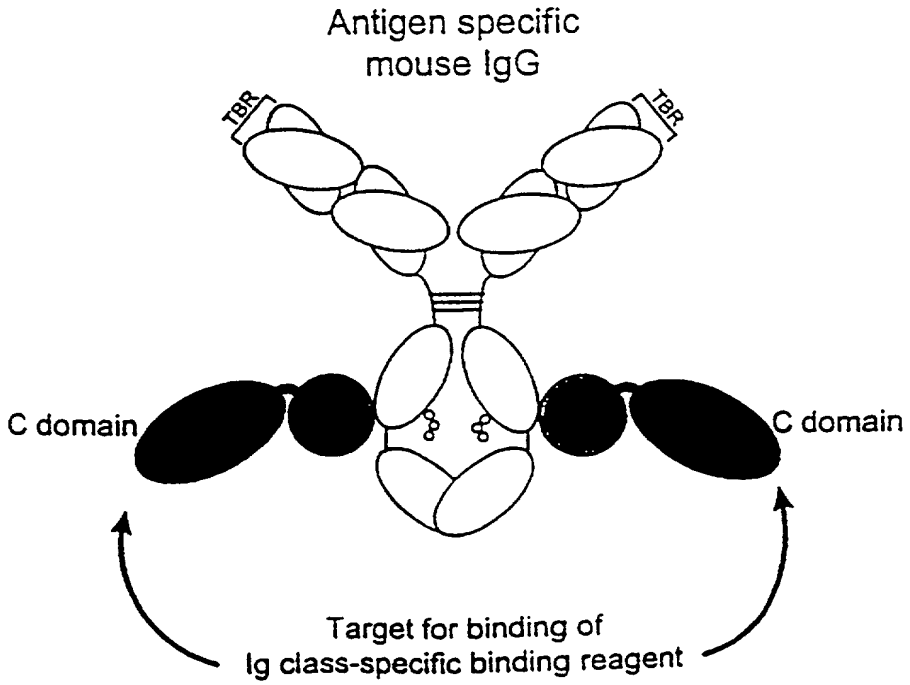
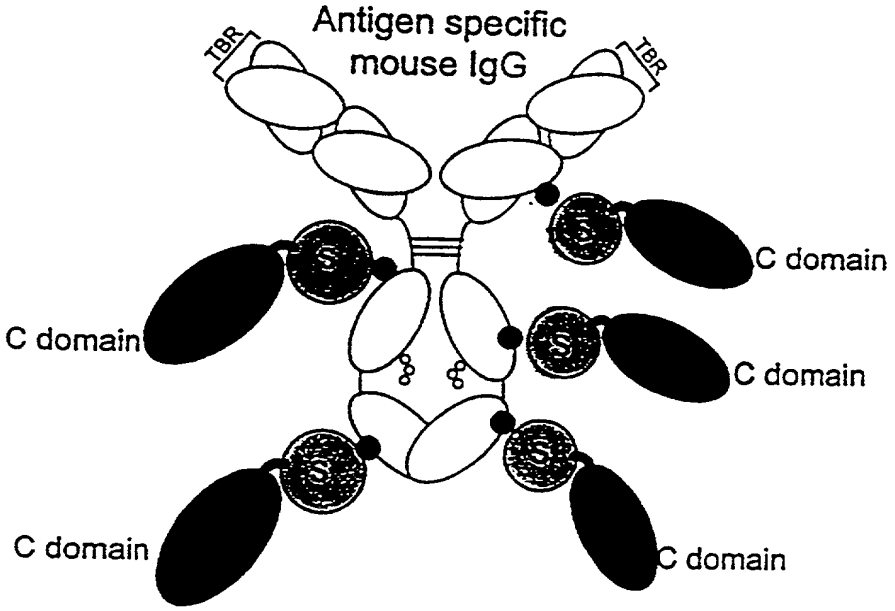


Figure 4

Complex formed between bifunctional molecule containing streptavidin and biotinylated mouse IgG



● = Biotin molecule

S = Streptavidin

Figure 5

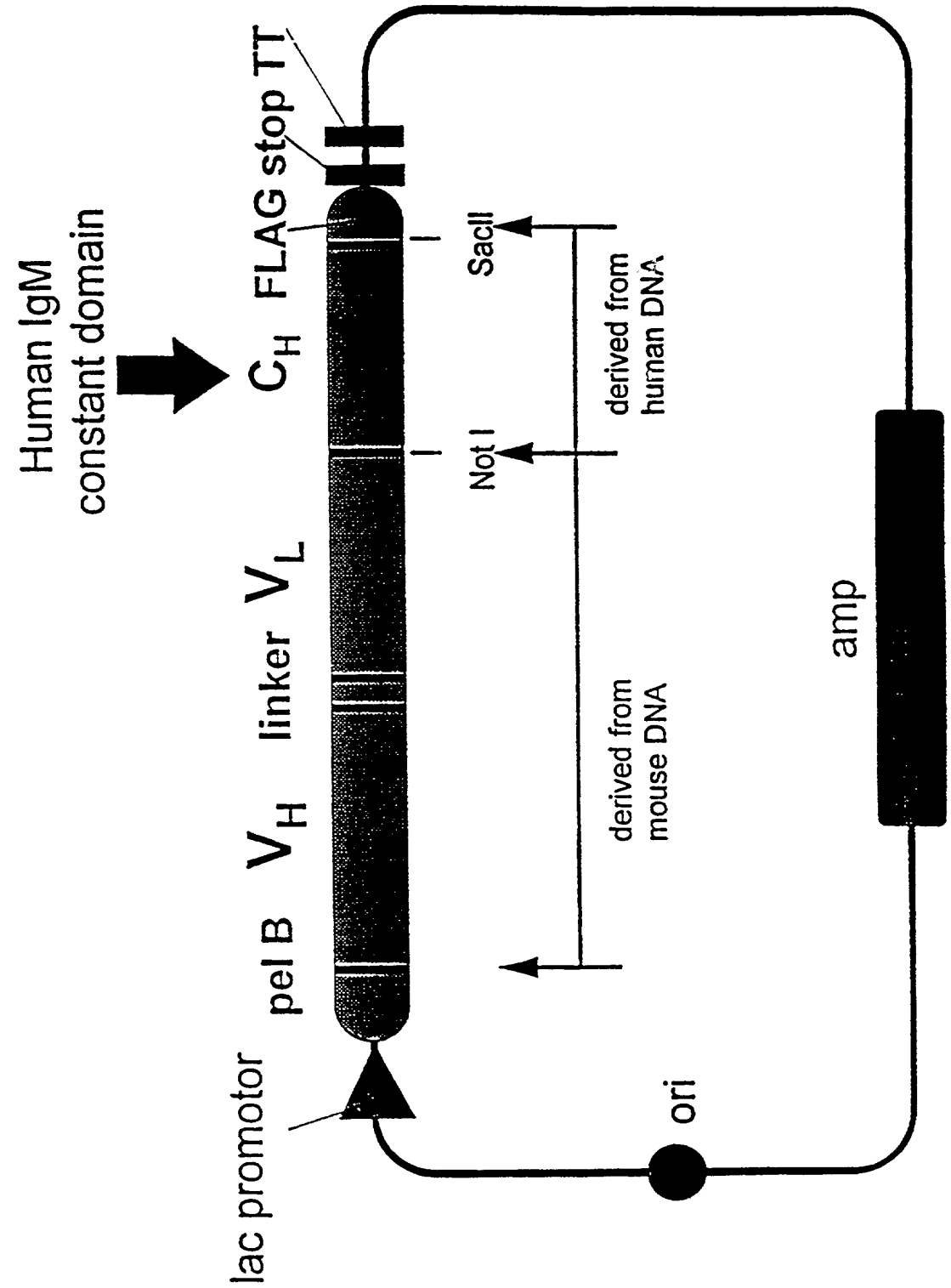

Human IgM
constant domain
C_H FLAG stop TT
SacII
Not I
derived from
human DNA
derived from
mouse DNA
pel B V_H linker V_L
amp
lac promotor
ori

Figure 6

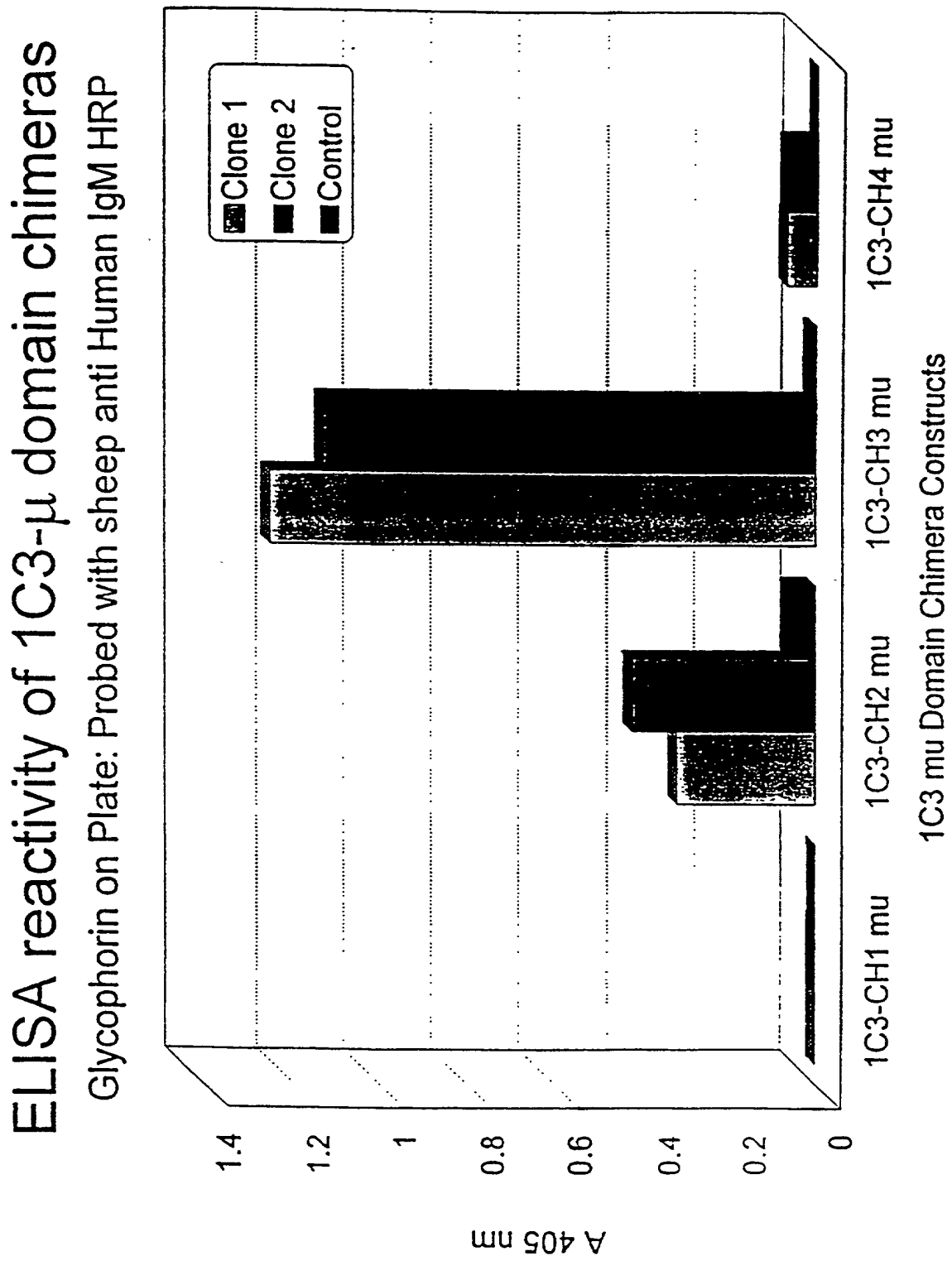

ELISA reactivity of 1C3-μ domain chimeras
Glycophorin on Plate: Probed with sheep anti Human IgM HRP
Clone 1
Clone 2
Control
A 405 nm
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1C3-CH1 mu
1C3-CH2 mu
1C3-CH3 mu
1C3-CH4 mu
1C3 mu Domain Chimera Constructs

Figure 7

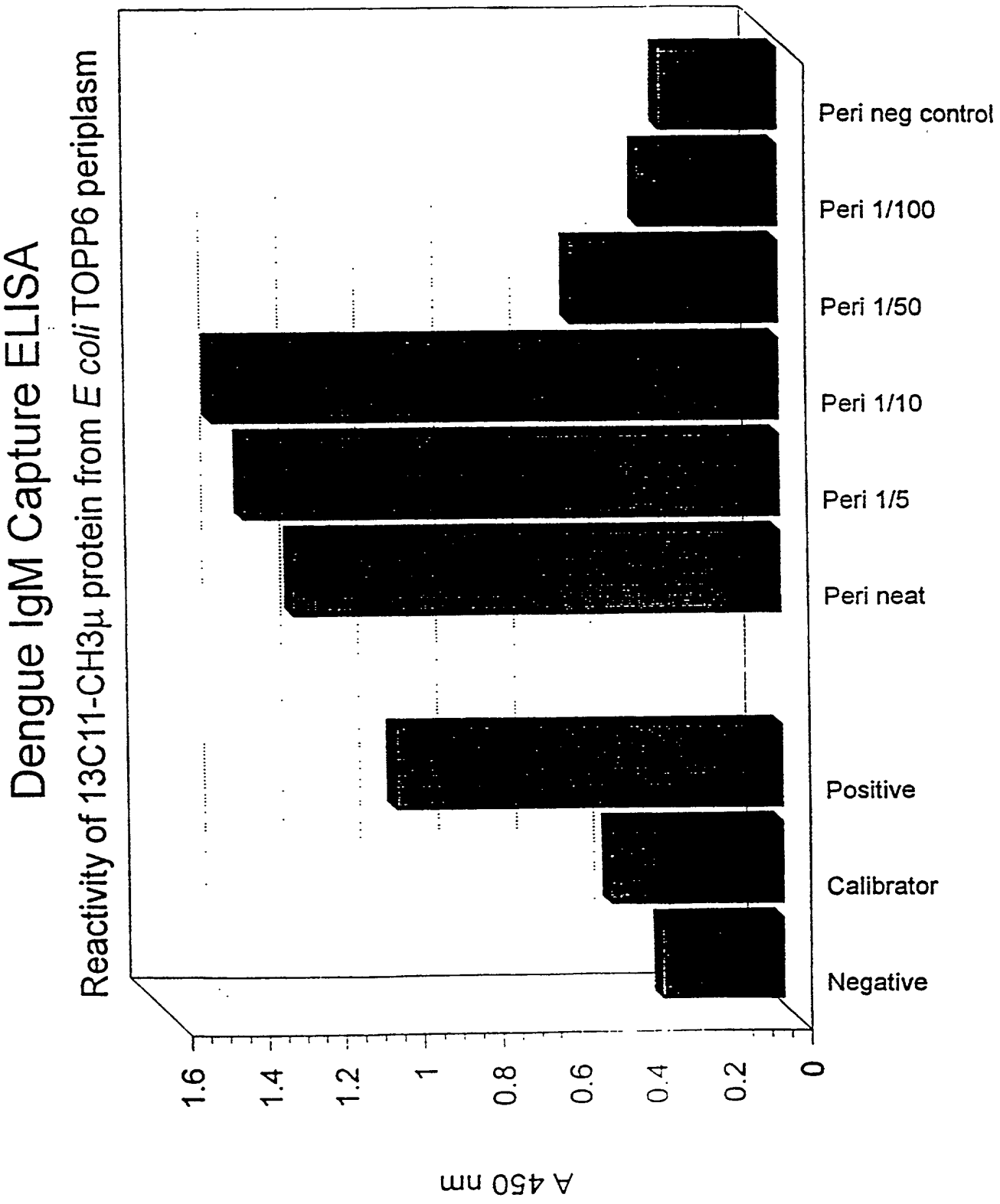

Dengue IgM Capture ELISA
Reactivity of 13C11-CH3μ protein from E coli TOPP6 periplasm
Peri neg control
Peri 1/100
Peri 1/50
Peri 1/10
Peri 1/5
Peri neat
Positive
Calibrator
Negative
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A 450 nm

Figure 8

```
      M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L   A
2353  ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG
      \-- Pel B-- -->                                               -

      A   Q   P   A   M   A   A   D   N   K   F   N   K   E   Q   Q
2401  GCC CAG CCG GCC ATG GCC GCG GAT AAC AAA TTC AAC AAA GAA CAA CAA
      --- Sfi 1    <N col >   < Start Fragment B

      N   A   F   Y   E   I   L   H   L   P   N   L   N   E   E   Q
2449  AAT GCT TTC TAT GAA ATC TTA CAT TTA CCT AAC TTA AAC GAA GAA CAA

      R   N   G   F   I   Q   S   L   K   D   D   P   S   Q   S   A
2497  CGC AAT GGT TTC ATC CAA AGC CTA AAA GAT GAC CCA AGC CAA AGC GCT

      N   L   L   A   E   A   K   K   L   N   D   A   Q   A   P   K
2545  AAC CTT TTA GCA GAA GCT AAA AAG CTA AAT GAT GCT CAA GCA CCA AAA
                                                    End Fragment B->

      S   D   P   A   A   A   D   Q   D   T   A   I   R   V   F   A
2593  AGT GAT CCC GCG GCC GCA GAT CAA GAC ACA GCC ATC CGG GTC TTC GCC
      < linker >    NotI  >  CH3 mu domain

      I   P   P   S   F   A   S   I   F   L   T   K   S   T   K   L
2641  ATC CCC CCA TCC TTT GCC AGC ATC TTC CTC ACC AAG TCC ACC AAG TTG

      T   C   L   V   T   D   L   T   T   Y   D   S   V   T   I   S
2689  ACC TGC CTG GTC ACA GAC CTG ACC ACC TAT GAC AGC GTG ACC ATC TCC

      W   T   R   Q   N   G   E   A   V   K   T   H   T   N   I   S
2737  TGG ACC CGC CAG AAT GGC GAA GCT GTG AAA ACC CAC ACC AAC ATC TCC

      E   S   H   P   N   A   T   F   S   A   V   G   E   A   S   I
2785  GAG AGC CAC CCC AAT GCC ACT TTC AGC GCC GTG GGT GAG GCC AGC ATC

      C   E   D   D   W   N   S   G   E   R   F   T   C   T   V   T
2833  TGC GAG GAT GAC TGG AAC TCC GGG GAG AGG TTC ACG TGC ACC GTG ACC

      H   T   D   L   P   S   P   L   K   Q   T   I   S   R   P   K
2881  CAC ACA GAC CTG CCC TCG CCA CTG AAG CAG ACC ATC TCC CGG CCC AAG

      G   A   A   D   Y   K   D   D   D   D   K   *
2929  GGc GCC GCG GAT TAT AAA GAT GAT GAT GAT AAA TAA GAA TTC AGC CCG
          Sac 2  ------------ FLAG -------------      Eco R1  <-------

2977  CCT AAT GAG CGG GCT TTT TTT TAA TTC ACT GGC CGT CGT TTT ACA ACG
      -------- TrpA terminator ----->
```

Figure 9

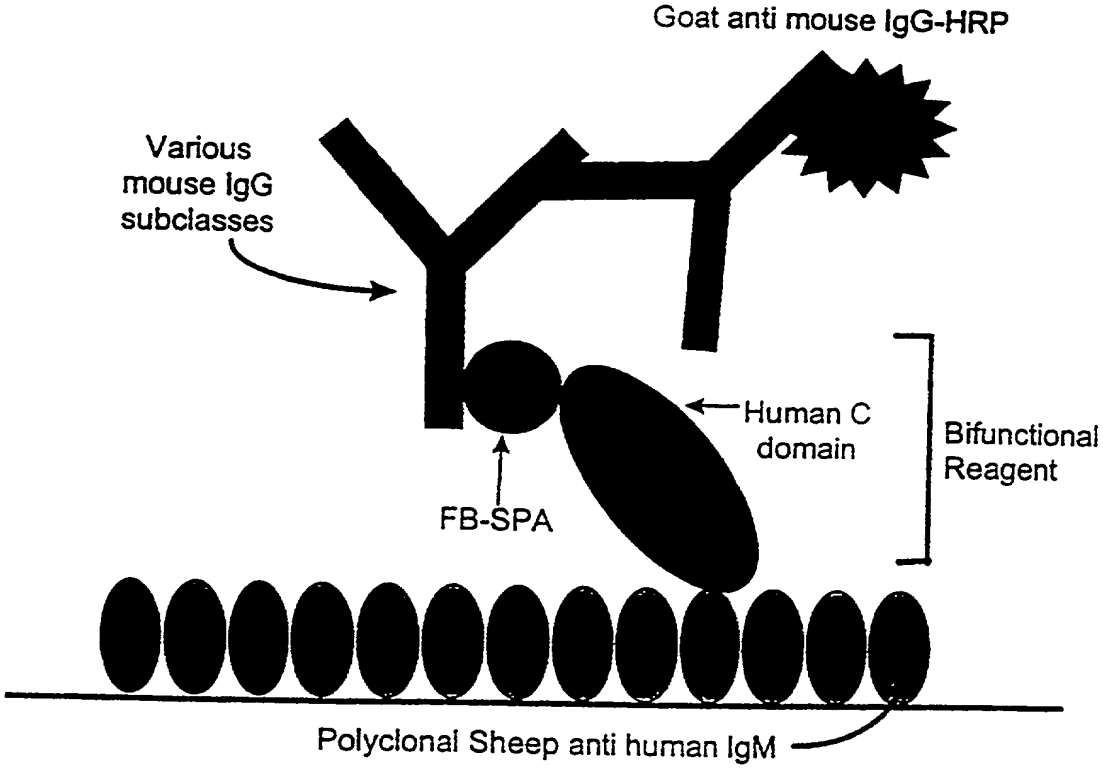
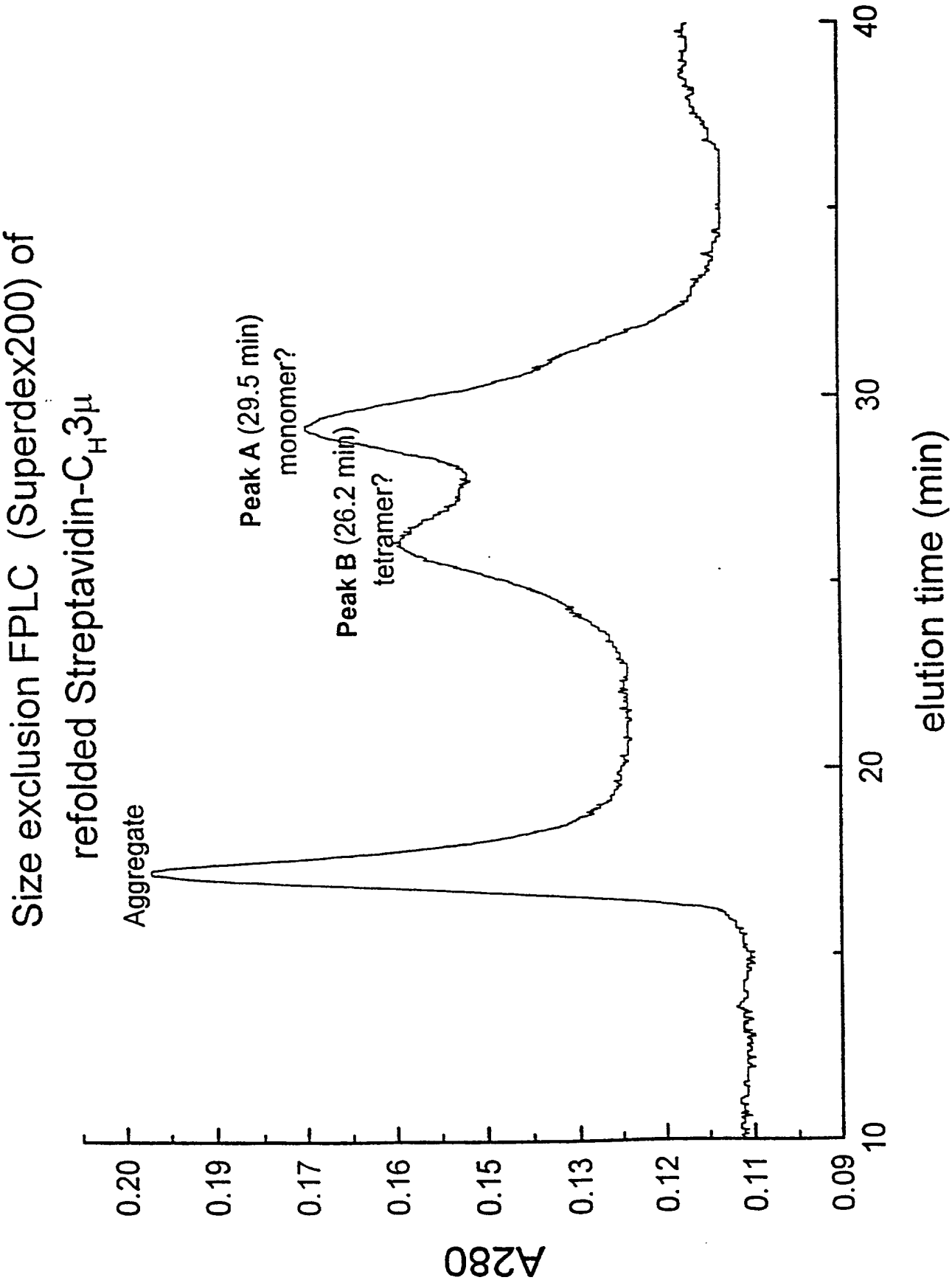

Goat anti mouse IgG-HRP
Various mouse IgG subclasses
Human C domain
Bifunctional Reagent
FB-SPA
Polyclonal Sheep anti human IgM

Figure 10

Sequence of expression cassette Str-$C_H3\mu$ in pGC vector

```
     M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L   A
ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCG
|-- Pel B-- -->                                                  -
     A   Q   P   A   M   A   E   A   G   I   T   G   T   W   Y   N
GCC CAG CCG GCC ATG GCC gag gcc ggc atc acc ggc acc tgg tac aac
--- Sfi I    <Nco I >----core streptavidin ---------------->
     Q   L   G   S   T   F   I   V   T   A   G   A   D   G   A   L
cag ctc ggc tcg acc ttc atc gtg acc gcg ggc gcc gac ggc gcc ctg

     T   G   T   Y   E   S   A   V   G   N   A   E   S   R   Y   V
acc gga acc tac gag tcg gcc gtc ggc aac gcc gag agc cgc tac gtc

     L   T   G   R   Y   D   S   A   P   A   T   D   G   S   G   T
ctg acc ggt cgt tac gac agc gcc ccg gcc acc gac ggc agc ggc acc

     A   L   G   W   T   V   A   W   K   N   N   Y   R   N   A   H
gcc ctc ggt tgg acg gtg gcc tgg aag aat aac tac cgc aac gcc cac

     S   A   T   T   W   S   G   Q   Y   V   G   G   A   E   A   R
tcc gcg acc acg tgg agc ggc cag tac gtc ggc ggc gcc gag gcg agg

     I   N   T   Q   W   L   L   T   S   G   T   T   E   A   N   A
atc aac acc cag tgg ctg ctg acc tcc ggc acc acc gag gcc aac gcc

     W   K   S   T   L   V   G   H   D   T   F   T   K   V   K   P
tgg aag tcc acg ctg gtc ggc cac gac acc ttc acc aag gtg aag ccg
                                                    -end core
     S   A   A   S   D   P   A   A   A   D   Q   D   T   A   I   R
tcc gcc gct agc gat ccc gcg gcc gca gat caa gac aca gcc atc cgg
strep-| -----< linker > <-Not I  >  |----$C_H3\mu$ domain
     V   F   A   I   P   P   S   F   A   S   I   F   L   T   K   S
gtc ttc gcc atc ccc cca tcc ttt gcc agc atc ttc ctc acc aag tcc

     T   K   L   T   C   L   V   T   D   L   T   T   Y   D   S   V
acc aag ttg acc tgc ctg gtc aca gac ctg acc acc tat gac agc gtg

     T   I   S   W   T   R   Q   N   G   E   A   V   K   T   H   T
acc atc tcc tgg acc cgc cag aat ggc gaa gct gtg aaa acc cac acc

     N   I   S   E   S   H   P   N   A   T   F   S   A   V   G   E
aac atc tcc gag agc cac ccc aat gcc act ttc agc gcc gtg ggt gag

     A   S   I   C   E   D   D   W   N   S   G   E   R   F   T   C
gcc agc atc tgc gag gat gac tgg aac tcc ggg gag agg ttc acg tgc

     T   V   T   H   T   D   L   P   S   P   L   K   Q   T   I   S
acc gtg acc cac aca gac ctg ccc tcg cca ctg aag cag acc atc tcc

     R   P   K   G   A   A   D   Y   K   D   D   D   D   K   *
cgg ccc aag ggc gcc gcg gat tat aaa gat gat gat gat aaa taa GAA
                 Sac 2   |------------FLAG-------------|     Eco

TTC AGC CCG CCT AAT GAG CGG GCT TTT TTT TAA TTC ACT GGC CGT CGT
```

Figure 11


Size exclusion FPLC (Superdex200) of refolded Streptavidin-$C_H3\mu$
Aggregate
Peak A (29.5 min) monomer?
Peak B (26.2 min) tetramer?
A280
0.20
0.19
0.17
0.16
0.15
0.13
0.12
0.11
0.09
10
20
30
40
elution time (min)

FLH Docket No.
674517-2001

DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(includes reference to PCT International Applications)

FROMMER LAWRENCE & HAUG LLP
File No.: 6745172001

As below named inventors, we each hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

We believe we are the original inventors (if plural, names are listed below) of. the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

BIFUNCTIONAL MOLECULES

the specification of which:
is attached hereto
was filed on _____ as
United States of America Serial No. _____
As the national phase of PCT Application No. PCT/AU98/01076
with amendments through DATE EVEN HEREWITH (if applicable, give details),

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a) - (d) or § 365 (b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Country (or PCT)	Application Number:	Filed (Day/Month/Year)	Priority Claimed: Yes	No
Australia	PP1110	24 December 1997	X	
Australia	PP5176	11 August 1998	X	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____
(Application Number) (Filing Date)

FLH Docket No.
674517-2001

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or § 365 (c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior United States or PCT International application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.:	Filed (Day/Month/Year)	PCT Application No.	Status (patented, pending, abandoned)
PCT	24 December 1998	PCT/AU98/01076	Pending (this application is the National Phase of the PCT)

I hereby appoint Thomas J. Kowalski, Registration No. 32,147, and FROMMER LAWRENCE & HAUG, LLP or their duly appointed associates, my attorneys or agents, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and to insert the Serial Number of the application in the space provided above, and specify that all communications about the application are to be directed to the following correspondence address:

Thomas J. Kowalski, Esq.
c/o FROMMER LAWRENCE & HAUG, LLP
745 Fifth Avenue
New York, NY 10151
FAX (212) 588-0500

Direct all telephone calls to: (212) 588-0800
to the attention of:
Thomas J. Kowalski

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTOR(S):

Signature: John L Atwell Date: 15 JUNE 2000

Full name of first inventor: **John Leslie ATWELL**
Residence: 7 Glenwerri Court Vermont South Victoria 3133 Australia AUX
Citizenship: Australia

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674517-2001

2-00 Signature: _____ Date: 9 June 2000

Full name of second joint inventor: **Peter Leonard DEVINE**
Residence: 135 Cribb Road Cairndale Queensland 4152 Australia AUX
Citizenship: Australia

Signature: _____ Date: 15 June 2000

3-00

Full name of third joint inventor: **Gregory COIA**
Residence: 73 Union Street Brunswick Victoria 3056 Australia AUX
Citizenship: Australia

Signature: _____ Date: 15 June 2000

4-00

Full name of fourth joint inventor: **Alexander Andrew KORTT**
Residence: 23 Upland Street Strathmore Victoria 3041 Australia AUX
Citizenship: Australia

Signature: _____ Date: 15/6/2000

5-00

Full name of fifth joint inventor: **Gillian Wendy PERRY**
Residence: 20 Gibbons Street Werribee Victoria 3030 Australia AUX
Citizenship: Australia

Signature: _____ Date: 8 June 2000

6-00

Full name of sixth joint inventor: **Peter Gregory BUNDESEN**
Residence: 15 Games Street Fig Tree Pocket Queensland 4069 Australia AUX
Citizenship: Australia

Post Office Address(es) of inventors [if different from residence]:

SEQUENCE LISTING

<110> CRC for Diagnostic Technologies

<120> Bifunctional molecules

<130> 91434

<140>
<141>

<160> 4

<170> PatentIn Ver. 2.1

SEQ ID NO: 1
<211> 203
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial sequence comprising sequence from Staphylococcal protein A fused to a sequence from human immunoglobulin

<400> 3

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Ala Gln Pro Ala Met Ala Ala Asp Asn Lys Phe Asn Lys Glu Gln Gln
            20                  25                  30

Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Asn Glu Glu Gln
        35                  40                  45

Arg Asn Gly Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala
    50                  55                  60

Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys
65                  70                  75                  80

Ser Asp Pro Ala Ala Ala Asp Gln Asp Thr Ala Ile Arg Val Phe Ala
                85                  90                  95

Ile Pro Pro Ser Phe Ala Ser Ile Phe Leu Thr Lys Ser Thr Lys Leu
            100                 105                 110

Thr Cys Leu Val Thr Asp Leu Thr Thr Tyr Asp Ser Val Thr Ile Ser
        115                 120                 125

Trp Thr Arg Gln Asn Gly Glu Ala Val Lys Thr His Thr Asn Ile Ser
    130                 135                 140

Glu Ser His Pro Asn Ala Thr Phe Ser Ala Val Gly Glu Ala Ser Ile
145                 150                 155                 160

Cys Glu Asp Asp Trp Asn Ser Gly Glu Arg Phe Thr Cys Thr Val Thr
                165                 170                 175

His Thr Asp Leu Pro Ser Pro Leu Lys Gln Thr Ile Ser Arg Pro Lys
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Gly Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys
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SEQ ID NO: 2
<211> 672
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial sequence comprising sequence from Staphylococcal protein A fused to a sequence from human immunoglobulin

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ttacctaact taaacgaaga acaacgcaat ggtttcatcc aaagcctaaa agatgaccca 180
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accaacatct ccgagagcca ccccaatgcc actttcagcg ccgtgggtga ggccagcatc 480
tgcgaggatg actggaactc cggggagagg ttcacgtgca ccgtgaccca cacagacctg 540
ccctcgccac tgaagcagac catctccggg cccaagggcg ccgcggatta taaagatgat 600
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cgttttacaa cg 672

SEQ ID NO: 3
<211> 270
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial sequence comprising sequence from *S.avidini* fused to a sequence from human immunoglobulin

<400> 1

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			20					25				30			
Gln	Leu	Gly	Ser	Thr	Phe	Ile	Val	Thr	Ala	Gly	Ala	Asp	Gly	Ala	Leu
		35					40					45			
Thr	Gly	Thr	Tyr	Glu	Ser	Ala	Val	Gly	Asn	Ala	Glu	Ser	Arg	Tyr	Val
	50					55					60				
Leu	Thr	Gly	Arg	Tyr	Asp	Ser	Ala	Pro	Ala	Thr	Asp	Gly	Ser	Gly	Thr
65					70					75					80
Ala	Leu	Gly	Trp	Thr	Val	Ala	Trp	Lys	Asn	Asn	Tyr	Arg	Asn	Ala	His
				85					90					95	
Ser	Ala	Thr	Thr	Trp	Ser	Gly	Gln	Tyr	Val	Gly	Gly	Ala	Glu	Ala	Arg
			100					105					110		
Ile	Asn	Thr	Gln	Trp	Leu	Leu	Thr	Ser	Gly	Thr	Thr	Glu	Ala	Asn	Ala
		115					120					125			
Trp	Lys	Ser	Thr	Leu	Val	Gly	His	Asp	Thr	Phe	Thr	Lys	Val	Lys	Pro
	130					135					140				
Ser	Ala	Ala	Ser	Asp	Pro	Ala	Ala	Ala	Asp	Gln	Asp	Thr	Ala	Ile	Arg
145					150					155					160
Val	Phe	Ala	Ile	Pro	Pro	Ser	Phe	Ala	Ser	Ile	Phe	Leu	Thr	Lys	Ser
				165					170					175	

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Thr Lys Leu Thr Cys Leu Val Thr Asp Leu Thr Thr Tyr Asp Ser Val
            180                 185                 190

Thr Ile Ser Trp Thr Arg Gln Asn Gly Glu Ala Val Lys Thr His Thr
        195                 200                 205

Asn Ile Ser Glu Ser His Pro Asn Ala Thr Phe Ser Ala Val Gly Glu
    210                 215                 220

Ala Ser Ile Cys Glu Asp Asp Trp Asn Ser Gly Glu Arg Phe Thr Cys
225                 230                 235                 240

Thr Val Thr His Thr Asp Leu Pro Ser Pro Leu Lys Gln Thr Ile Ser
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Arg Pro Lys Gly Ala Ala Asp Tyr Lys Asp Asp Asp Asp Lys
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SEQ ID NO: 4
<211> 864
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Artificial sequence comprising sequence from *S.avidini* fused to a sequence from human immunoglobulin

<400> 2

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accgcgggcg ccgacggcgc cctgaccgga acctacgagt cggccgtcgg caacgccgag 180
agccgctacg tcctgaccgg tcgttacgac agcgccccgg ccaccgacgg cagcggcacc 240
gccctcggtt ggacggtggc ctggaagaat aactaccgca acgcccactc cgcgaccacg 300
tggagcggcc agtacgtcgg cggcgccgag gcgaggatca acacccagtg gctgctgacc 360
tccggcacca ccgaggccaa cgcctggaag tccacgctgg tcggccacga caccttcacc 420
aaggtgaagc cgtccgccgc tagcgatccc gcggccgcag atcaagacac agccatccgg 480
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tgcctggtca cagacctgac cacctatgac agcgtgacca tctcctggac ccgccagaat 600
ggcgaagctg tgaaaaccca caccaacatc tccgagagcc accccaatgc cactttcagc 660
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gccgcggatt ataaagatga tgatgataaa taagaattca gcccgcctaa tgagcgggct 840
ttttttaat tcactggccg tcgt                                        864
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